THE NEUROPHARMACOLOGY OF SOME INJECTABLE ANAESTHETICS STUDIED IN THE LAMPREY

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Preface

The experimental work described herein was performed under the supervision of Professor F. Alexander and Dr. R.J. Martin during the tenure of a University Demonstratorship in the Department of Veterinary Pharmacology. I hereby declare that the experimental work is entirely my own, that this dissertation is my own composition and that it has not previously been submitted in whole or in part to this or any other University.

The substance of Sections III and V has been published

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and in accordance with Regulation 2.4.11 a reprint is appended hereto.

Katharine Deirdre Cullen

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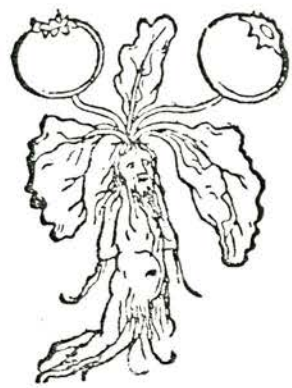
The work was made possible by the award of a Project Grant by the Wellcome Trust to Dr. R.J. Martin. Glaxovet kindly supplied the vehicle used commercially in Saffan free of charge.

To the memory of my father

M. D. XXXvij.



CONTENTS

LIST OF TABLES

LIST OF FIGURES

Summary

A comparative study of four anaesthetic drugs has been undertaken with the object of improving the understanding of their neuropharmacology. Pentobarbitone, ketamine (Vetalar*), alphaxalone (Saffan*) and metomidate have been selected to represent the types of compound currently in clinical use. With a more extensive knowledge of the drugs at their disposal clinicians will be better able to exploit the different properties of individual drugs.

Intracellular recordings have been made from identified bulbar reticulo-spinal (Müller) cells in the medulla of lamprey ammocoetes. Responses to iontophoretically applied transmitters have been measured as changes in membrane potential and input resistance. Bath application of anaesthetics resulted in dose-dependent alterations in the responses to γ-aminobutyric acid (GABA), glycine and glutamate. These amino acids are transmitters thought to be of major importance in central nervous mechanisms.

The suitability of the lamprey preparation for a pharmacological study of this nature is discussed. All four drugs have been tested and shown to anaesthetise lampreys; although pentobarbitone was effective only when administered by injection.

Anaesthetic effects on the spontaneous synaptic activity recorded from Müller cells have been measured: EPSPs were decreased in frequency by pentobarbitone (10^{-4}M) and, after a transitory increase, by alphaxalone ($1-3 \times 10^{-5}$M). Sub-anaesthetic concentrations of ketamine ($<10^{-5}$M) stimulated activity in the one preparation tested. High concentrations (c.10^{-3}M) of all drugs suppressed all spontaneous activity: below this concentration

metomidate had little effect. IPSPs were more labile than EPSPs: they were reduced in frequency by all drugs at anaesthetic or supra-anaesthetic concentrations, and this reduction was longer lasting than that of EPSPs.

GABA responses were potentiated by pentobarbitone (1-3 x 10^{-4}M) and prolonged by ketamine (3.7 x 10^{-4}M); but depressed by high concentrations (10^{-3}M) of all drugs, as well as by anaesthetic concentrations of alphaxalone (1-3 x 10^{-5}M).

Glycine responses were depressed by alphaxalone (1-3 x 10^{-5}M) and by supra-anaesthetic concentrations of ketamine (3.7 x 10^{-4}M) and metomidate (1.8 x 10^{-3}M). No drug potentiated the glycine responses.

Anaesthetic effects on glutamate responses were more variable and much less striking than were those on GABA and glycine responses. Only pentobarbitone and ketamine had consistent dose-dependent inhibitory effects. No potentiation of glutamate responses was observed.

In the absence of an effect common to the four anaesthetics, it is concluded that neither potentiation nor inhibition of all GABA, glycine or glutamate responses is an essential feature of anaesthesia. However, effects comparable to those described here may contribute to the overall clinical picture during anaesthesia of higher vertebrates. Attention is drawn to the difficulty of predicting the consequences of effects observed on a single component of a polysynaptic pathway. The findings do not support the notion that all anaesthetic agents act on biological membranes by a single mechanism.

Introduction

> ...and as there is no greater inconvenience to any Arteficer, than not to know his tools that he must work with...
>
> Porta (1561)

Porta referred to the necessity of botanical knowledge to the practising magician, but the thought is equally applicable to the modern anaesthetist, who can practise his art efficiently and safely only if he has a good understanding of the drugs at his disposal. With this in mind, I present here the initial results of a comparative study of the neuropharmacology of some injectable anaesthetic agents. The study falls into three sections:

i) investigation of the capability of each anaesthetic to induce anaesthesia in lamprey ammocoetes (Section III); a necessary screening experiment;

ii) measurements of anaesthetic-induced changes in the spontaneous synaptic activity recorded from reticulo-spinal cells (Section IV);

iii) observations of the effects of anaesthetics on the responses of identified cells to inhibitory (Section V) and excitatory (Section VI) amino-acid neurotransmitters.

Four drugs have been considered in detail: these were selected to represent the wide range of compounds currently available for intravenous administration. The project was designed rather to further the understanding of those drugs currently in clinical use in order that they may be used more effectively, than to study the mechanisms of anaesthesia. Closely related but non-

anaesthetic compounds have therefore not been included, nor have racemic mixtures been separated to investigate the different properties of their components.

Evidence of interactions between the anaesthetics and three putative amino-acid neurotransmitters has been sought. These amino acids are thought to be of major importance in central nervous mechanisms; although they are numerically but a small sample of current transmitter candidates, and represent only one category of such substances.

A single preparation has been used throughout: bulbar reticulospinal cells in the brain-stem of a simple chordate, the lamprey. Insofar as they are understood, neurotransmitter mechanisms in the lamprey seem similar to those in mammalian brains, so the simpler animal is acting in this study as a model for the more complex.

It should be stressed at the outset that the bulbar reticulospinal cells are not directly involved in the control of consciousness although they are located in the reticular formation, and therefore no conclusions can be drawn as to the mechanisms by which anaesthesia is induced. Secondly, although two of the amino acids used, glycine and L-glutamate, are good candidates for the roles, the natural transmitters acting on these cells have yet to be positively identified (Matthews & Wickelgren, 1979a,b; Gold & Martin, 1982b). However, comparison of the effects of the drugs on the cells' responses, and correlation with their effects on spontaneous synaptic activity, enable comment to be made on the side effects reported by clinicians. The question then arises as to

whether the results cast any light on the management or medication most likely to lessen the impact of these side effects in the peri-operative period. Any such extrapolation from the lamprey to higher vertebrates assumes comparability of the cell systems studied, and of individual cell responses to the drugs used. No major discrepancy has yet come to light.

SECTION I

Prologue

General Anaesthetics: an historical preamble

> ...and the Lord God caused a deep sleep to fall upon Adam, and he slept: and he took one of his ribs, and closed up the flesh instead of thereof...
>
> Genesis II:21

This, probably the first reference to a surgical operation performed under some form of anaesthesia, testifies to the prevalence of the concept of anaesthesia at the time the book of Genesis was compiled. Anaesthesia does not, as many modern writings imply, date from the mid-19th Century (although its widespread application probably does) as further quotations also illustrate:

> ...il donnait au malade une préparation de chauvre (Ma-yo), et, au bout de quelques instants, il devenait aussi insensible que s'il eût été plongé dans l'ivresse ou privé de vie. Alors, suivant le cas, il pratiquait des ouvertures, des incisions, des amputations, et enlevait la cause du mal; puis il rapprochait les tissus par des points de suture et y appliquait des liniments. Après un certain nombre de jours (au bout d'un mois, suivant les Annales des Hân postérieurs), le malade se trouvait rétabli sans avoir eprouvé, pendant l'operation, la plus légère douleur...
>
> Biography of Hoa-tho, eminent physician in the Wei dynasty, 220-230 A.D., in a 16th Century Chinese account of medicine ancient & modern.
>
> Transl. Stanislas Julien, (1849)

The historical accuracy of this account is uncertain because the biography is undated, but the idea of anaesthesia must have been current in the 16th Century. (Ma-yo was an extract of the leaves of Cannabis indica taken with wine.)

> ...some persons boil down the roots in wine to a third,strain it, and put it away, using one cyanthus in the case of persons suffering from insomnia or severe pain, or those about to be cut or cauterised, when they wish to produce anaesthesia...
>
> ...wine is also made from the bark of the root without boiling; for this purpose put three minae into one metretes of sweet wine. Three cyathi of this should be

> given to persons about to be cut or cauterised...They do not feel the pain on account of the ensuing stupor...

Dioscorides, a herbalist and physician of the 1st Century A.D., here used the term "anaesthesia" in its modern sense (it was coined independently by Holmes in 1846). The two extracts from his writings were cited by Randolph (1905) in an exhaustive study of ancient references to Mandragora. Randolph concluded after examining the available sources that mandragora, whose active ingredients include atropine, hyoscine and hyoscyamine, was the principal, indeed almost the only anaesthetic of antiquity, and that it was used by physicians at least as early as the 1st Century A.D. However, there is only presumptive evidence that mandragora was in fact used; Randolph found no mention of it in any description of a specific operation. The reason why anaesthesia was not more widely practised must be a matter for conjecture, but it is pertinent that the dangers attendant on the use of available agents were well recognised. Other agents mentioned in the ancient literature include tears of poppy (opium), Hyoscanthus, Morum (mulberry) and Lactuca silvestris (wild lettuce) (Tallmadge, 1946).

Interestingly, although the mandrake has become associated with a wealth of legends (some of them, such as the various digging ceremonies, probably transferred from other plants), two species of mandragora have been identified and shown to have genuine anaesthetic properties. Richardson had some success in reproducing the ancient wine of mandragora:

> ...the wine of the mandragora is a general anaesthetic of the most potent quality. The action no doubt depends on the presence of an alkaloid which is like, if not identical with, atropine, and from it an alkaloid could be extracted which might be used medicinally, and which

> would, I have no doubt, be one of the most active anaesthetics we have yet discovered. From the circumstance that the heart continues to beat after the respiration has ceased we may infer that as a general anaesthetic the alkaloid might, under necessity, be once more employed, as in the olden time, to deaden the pain of a surgical operation, and that, too, with comparatively little risk to life...
>
> Richardson (1888)

It is notable that all the agents mentioned so far were to be administered orally or per rectum rather than by inhalation. The first mention of gaseous anaesthesia was in Davy's account of the properties of nitrous oxide:

> ...as nitrous oxide in its extensive operation appears capable of destroying physical pain, it may probably be used with advantage during surgical operations in which no effusion of blood takes place...
>
> Davy (1800).

However, not only did Davy fail to exploit this observation, he also frustrated Hickman's attempts in 1826 to introduce carbon dioxide anaesthesia. In like manner, his patron Beddoes failed to exploit ether although he recorded its anaesthetic properties in 1818. Both ether and nitrous oxide were used only frivolously for their stimulant properties until the 1840's.

Intravenous techniques followed later, but the first intravenous injection of a narcotic drug had been made two centuries previously by Sir Christopher Wren:

> ...the most considerable experiment I have made of late, is this; I injected Wine and Ale into the mass of blood in a living dog, by a vein, in good quantities, till I made him extremely drunk, but soon after he pissed it out...it will be too long to tell you the effects of opium, scammony, and other things which I have try'd this way. I am in further pursuit of the experiment, which I take to be of great concernement, and what will give great light to the Theory and Practice of Physick...
>
> Wren (1656).

No description of the effects of opium followed but Boyle, an

eye-witness to this the first documented intravenous injection, later described the effects of the injection of a warm solution of opium in sack:

> ... (Wren) conveyed a small dose of the solution or tincture into the opened vessel, whereby getting into the mass of blood (some quantity of which it 'tis difficult to avoid shedding in the operation) it was quickly, by the circular motion of that, carried to the brain, and other parts of the body: so that we had scarce untied the dog (whose four feet it had been requisite to fasten very strongly to the four corners of the table) before the opium began to disclose its narcotick quality, and almost as soon as he was on his feet, he began to nod with his head, and faulter and reel in his pace, and presently after appeared so stupified, that there were wagers offered his life could not be saved...
>
> Boyle (quoted in a memoir Parentalia compiled by Wren's son and published by his grandson: Wren, 1750).

The dog however was saved by dint of being "whipped up and down a neighbouring garden until he came to himself", and was subsequently used for further experiments until, being a notoriety, he was stolen. From this "invention" developed the technique of blood transfusion (King & Lower reported a successful transfusion from sheep to human in 1667), but routine intravenous administration of medicinal substances did not immediately follow. Wren's technique entailed exposing and ligaturing a large superficial vein, before slitting it and inserting a quill or other slender pipe by which the solution was injected. The description is of quills fastened to a bladder "in the manner of clyster pipes".

Is it coincidence that the development of intravenous anaesthesia followed closely after the invention of the hypodermic needle and syringe?

Theories of Anaesthesia

> ...it would be easy to form theories referring the action of blood impregnated with nitrous oxide, to its power of supplying the nervous and muscular fibre with such proportions of condensed nitrogene, oxygene and light or ethereal fluid, as enabled them more rapidly to pass through those changes which constitute their life: but such theories would be only collections of terms derived from known phaenomena and applied by loose analogies of language to unknown things...
>
> Davy, Researches on nitrous oxide (1800)

The literature concerning anaesthetics and the mechanisms by which they may act is voluminous, and has been comprehensively reviewed at intervals during the past century (recent reviews include Seeman, 1972; Kaufman, 1977; Richards, 1978; Roth, 1979; Gage & Hamill, 1981; Franks & Lieb, 1982). By this brief introduction I aim to set the scene for the present investigation, not to retell a story which has been told many times before and at far greater length than is appropriate here.

Bernard (1875) was the first to propose a unitary theory whereby the actions of all agents depressant to the central nervous system might be explained; and thereby set a trend for many subsequent theories. The philosophical desire for a unitary theory was based partly on the recognised diversity of compounds with anaesthetic actions (these even including the chemically unreactive rare gases xenon and krypton), and partly on the assumption that there was a single anaesthetic state induced by all anaesthetic drugs. Because of the chemical diversity of the drugs a non-specific mechanism was sought rather than a specific "anaesthetic receptor". Anaesthetic research has proceeded on more than one level, and only recently has it begun to be possible to assimilate

the many strands into a single picture. This picture is as yet far from complete and will remain so until such time as mechanisms of consciousness are much better understood.

Overton in 1899 and Meyer in 1901 independently were the first to correlate the anaesthetic potencies of a series of alcohols with their partition coefficients between water and olive oil. This was the first statement of the "lipid theory", a theory which has remained current in a refined form to the present day. However, its limitations have been recognised. For example Butler (1950), despite admitting Ferguson's argument (1939) that the correlation was considerably better, as well as more appropriate, if the thermodynamic activities of the drugs in the various phases in equilibrium were used in place of their concentrations, was at pains to show that such correlations could do no more than suggest that the site of action of anaesthetics was in an apolar region of the cell, be it a protein molecule or a membrane lipid, and offered no mechanism by which the depressant effect was exerted. Butler also pointed out that Meyer (1936) had applied the lipid theory only to "indifferent narcotics" or the "alcohol group", a fact that was sometimes overlooked, and that he had recognised the possibility that other compounds may depress brain function by different means.

The lipid theory assumed the status of a hypothesis of anaesthesia only when Mullins (1954) proposed that anaesthetics act by expanding the surface membrane lipid, so disordering its structure that the functioning of enzymes, ion channels and receptors was compromised. A more recent general hypothesis concerns the demonstrable ability of many anaesthetics to increase

the "fluidity" of lipid membranes; by this was meant the freedom of movement of the apolar lipid chains which are usually aligned in a plane normal to the membrane surface. An increase in fluidity can be detected by magnetic resonance techniques (see Metcalfe, Hoult & Colley, 1974; Kaufman, 1977), and there is some evidence that anaesthetic steroids and barbiturates can be distinguished from their non-anaesthetic relatives by their ability to cause such an increase (e.g. Lawrence & Gill, 1975; Novak & Swift, 1976). Alterations in fluidity must alter the constraints on the protein molecules that constitute the membrane ion "channels", and are therefore likely to affect their physical properties: the ion permeabilities and the kinetics of opening and closing the channels. It has however yet to be demonstrated that an increase in membrane fluidity is either necessary or sufficient for anaesthesia to occur; and any evidence for the various mechanistic possibilities is lacking.

Recently the lipid interacting with a local anaesthetic has been identified less with the bulk of the surface membrane than with the annulus immediately surrounding the sodium channel (Lee, 1976). Application of this concept to general anaesthetics has the attraction that there is the potential to incorporate stereo-specific effects of a drug, and tissue-specific effects (see Metcalfe, Hoult & Colley, 1974). As Gage & Hamill (1981) made clear however, no distinction can confidently be made between an effect on the lipid annulus and one on a hydrophobic part of the protein constituting the ion channel. The effect of an anaesthetic molecule on a particular membrane protein or lipid will depend on the

latter's composition; insufficient is known as yet about the detail of either. Arguments have raged as to the most appropriate lipid to use in such studies (see Kaufman, 1977) but the crucial effect of the environment of the lipid has often been ignored. It is likely that a lipid that forms an integral part of the cell membrane is so constrained by the cell architecture and by the adjacent cytoplasm that its properties differ from those of the same lipid either in bulk phase or as a thin "black" membrane.

At this point the proponents of the modified lipid theory met the neurophysiologists and neuropharmacologists on common ground. There had been a parallel development of anaesthetic theories concerned with events at the cellular rather than the molecular level, springing from Sherrington's observation (1906) that spinal reflexes were more readily depressed by anaesthetics (by a factor of 10) than was impulse conduction in peripheral nerves. Attention was thus directed to the synapse as the likely site of action, and detailed studies have been performed on accessible synapses: notably the neuro-muscular junction, invertebrate neural ganglia, autonomic and dorsal root ganglia. Latterly the development of brain slice preparations has allowed extracellular studies on mammalian brain cells. Well controlled intracellular studies have been carried out on cultures of mammalian brain and spinal cord.

The mechanism of the selective effect of anaesthetics on synaptic transmission has yet to be fully elucidated. Larrabee & Posternak (1952), showing the diverse effects of different agents on transmission through sympathetic ganglia, suggested anaesthetic

blockade of conduction in the fine presynaptic nerve terminals as an economical unitary mechanism. Current opinion is that there is no good evidence for such a theory (Richards, 1978, 1982). There is no reason to suppose that all anaesthetic agents must act by a single mechanism, and it has long been known that some differ in their pharmacological effects. For example, Derbyshire et al (1936) reported that ether and pentobarbitone had different effects on the cortical electro-encephalogram (EEG). The accumulating evidence for both pre- and postsynaptic effects is considered more fully in connection with pentobarbitone (p.20) and in Sections V & VI.

Although most theories assume that the surface membrane is that principally affected by the anaesthetic process, an apolar site is also provided by the extensive intra-cellular membranes. Krnjević (1975) emphasised this when proposing that anaesthesia resulted from a reduced mitochondrial uptake of calcium: the consequent rise in free intracellular calcium should promote transmitter release at synapses and hyperpolarise the postsynaptic membrane by increasing the membrane permeability to potassium. The possible role of calcium in the anaesthetic process has been a continuing thread running through anaesthetic research, and from time to time has been invoked to explain experimental findings (e.g. Morris, 1978). Should intracellular accumulation of free calcium be an intrinsic part of the mechanism, it would be expected to have also quite other and far-reaching consequences.

An anaesthetic-dependent calcium-induced loss of respiratory control in mitochondria has been reported, associated with anaesthetic concentrations (10^{-5} to 10^{-4} M) of alphaxalone (Smith,

Sweetman & Esmail,1974; Esmail, Smith & Sweetman, 1974). Sweetman & Esmail (1975), found that mitochondrial uptake of radio-labelled calcium was reduced after treatment with either alphaxalone (Althesin) or hexobarbitone. Nonetheless, Pennefather, Puil & Quastel (1980) measured a reduction in depolarisation-secretion coupling at the mouse neuro-muscular junction during exposure not only to alphaxalone and other anaesthetic steroids (such as progesterone), but also to the non-anaesthetic isomer Δ^{16} - alphaxalone. Whilst this effect may not therefore be relevant to mechanisms of anaesthesia, it must be taken into account in considering the results of Section IV. Calcium has also been implicated in the presynaptic actions of pentobarbitone (Krnjević, 1975) and ketamine.

A unitary theory of this type goes part-way towards fulfilling the requirement that anaesthetic depression be greater at synapses, but it does not explain the different susceptibilities shown by different synapses. Other theories have attributed anaesthesia to drug actions at specific membrane receptors for neurotransmitters. Some of this evidence is considered in Sections V & VI. Such theories, whilst they may account for differences in drug effects, do not propose any molecular mechanism that has adequate experimental support (Gage & Hamill, 1981).

Reviewing the more recent studies, Franks & Lieb (1982) have suggested that the various lipid theories are less than satisfactory. This was not recognised previously because earlier studies employed anaesthetic concentrations greater than those

encountered during surgical anaesthesia. Clinically relevant concentrations have only small effects on membrane dimensions, and no detectable effect on the membrane fluidity. Franks & Lieb have pointed out that most of the experimental data, such as that concerning the influence of temperature and the reversal of anaesthesia by pressure, can be explained equally well by a simple model in which an anaesthetic binds to a functional membrane protein.

Many have now abandoned the concept of a truly unitary mechanism of anaesthesia. Not only are there recognised differences between drugs in the progression of clinical states, but anaesthetics differ in their effects on the EEG and at the level of individual synapses. Furthermore, some mixtures of anaesthetics have been shown to have non-additive effects on sleeping times in mice (Richards & White, 1981). Insofar as the lamprey preparation can be considered an appropriate model of the mammalian CNS, the results of this investigation constitute additional evidence against a unitary hypothesis.

The Drugs

> A Sleeping Apple: for it is made of opium, Mandrake, juice of Hemlock, the seeds of Henbane; and adding a little Musk, to gain an easier reception of the smeller: these being made up into a ball, as big as a man's hand can hold, and often smelt to, generally close the eyes, and bind them with a deep sleep.
>
> Porta (1561)

Four drugs were selected to represent the types of compound currently in veterinary clinical use for the induction of anaesthesia by intravenous injection. The drugs are chemically very different and although they share the ability to induce anaesthesia, the resultant clinical states show differences, as do the side effects reported. In this initial survey the compounds used were:

pentobarbitone sodium - a barbiturate,

ketamine hydrochloride - a phenylcyclohexylamine,

alphaxalone/alphadolone - a mixture of two derivatives of the steroid pregnanedione,

metomidate hydrochloride - an imidazole derivative.

It was expected that the drugs would differ in their effects on the reticulo-spinal cells. These cells have essentially a motor function (p.39), and marked differences are reported by clinicians in the degree of muscle tone retained during anaesthesia and in the incidence of excitatory motor effects such as twitching, myoclonus and convulsions, according to which drug has been used.

Barbiturates

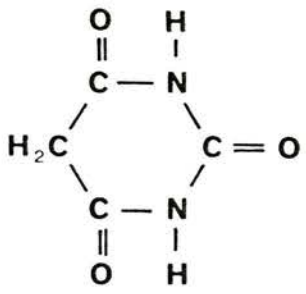
The barbiturates were among the earliest drugs to be developed for intravenous anaesthesia. The parent compound, barbituric acid,

itself had no anaesthetic activity, but its 5:5 diethyl derivative was introduced into medicine as a hypnotic by Fischer & von Mering in 1903. Pentobarbitone (Figure I-1) was introduced into medical practice in 1930 and to veterinary practice in 1931 (Kreutzer, 1931). Since then it has been extensively used both in clinical practice (e.g. Wright, 1934, 1938) and as an anaesthetic for experimental animals, but it has now been superceded for most purposes by shorter acting barbiturates such as thiopentone and methohexitone, or by non-barbiturate anaesthetics. Because of the widespread use of pentobarbitone in experimental studies there is a wealth of information available concerning its neuropharmacology, and this was the main reason for its selection in the present investigation. A comprehensive review of the published work relevant to this study is not realistic, but some of the more important points will be considered briefly. This will serve also to trace the development of ideas about anaesthetic actions on synaptic transmission.

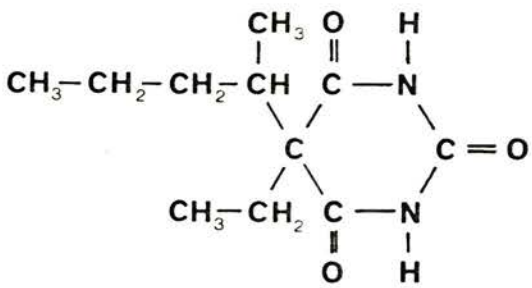
EEG studies The onset of sleep has long been known to be associated with a change in the EEG pattern from one of low voltage fast waves, when cortical neurones are firing out of phase, to slower synchronised waves of greater amplitude. Brazier & Finesinger (1945) reported a similar change a few minutes after barbiturate administration had induced unconsciousness. They also noted a transient period during both induction and recovery, when there was high voltage fast EEG activity. Derbyshire et al (1936) had earlier reported few changes in the spontaneous and evoked cortical potentials following administration of pentobarbitone,

Figure I-1

Structures of barbituric acid and its derivative pentobarbitone



Barbituric acid



Pentobarbitone

although the cortical waves increased in amplitude, implying some synchronous activity of cells. During anaesthesia the spontaneous waves were infrequent but sciatic stimulation evoked a big electrical response. Schlag, Quadens & Kridellca (1956) observed that pentobarbitone (15-30mg/kg), unlike ether and α-chloralose, depressed the spontaneous activity of the somatosensory cortex, the hippocampus and the reticular formation without affecting evoked activity in the cortex. Sparing of the sensory paths has been observed repeatedly with techniques that have looked at different parts of the pathway (e.g. Forbes & Morison, 1939; Mark & Steiner, 1958; Morris, 1978). The latter measured transmission in the cuneate nucleus, one of the relay stations en route to the sensory cortex.

The reduced spontaneous activity and the more synchronised EEG waves indicated cortical depression. More recent observations of reduced responsiveness of cortical neurones to the excitatory transmitters glutamate and acetylcholine (Richards, 1972, 1974; Richards & Smaje, 1976; Smaje, 1976) have provided supportive evidence.

Effects at synapses Not all early experiments recorded increased or spared synaptic transmission: Brooks & Eccles (1947) found that all components of the monosynaptic reflex path through the spinal cord were blocked by pentobarbitone, but they were using supra-anaesthetic doses (30-120mg/kg), concentrations sufficient to block axonal impulse conduction although not to prevent generation of a large synaptic potential. Larrabee & Posternak (1952) in classic and oft quoted experiments showed that pentobarbitone concentrations

equivalent to those known or calculated to obtain in blood during surgical anaesthesia, blocked transmission in sympathetic ganglia but were not sufficient to block axonal conduction. The extent of this selectivity varied between anaesthetics. Exley (1954) also found autonomic ganglionic depression, especially from amylobarbitone, but noted in comparing three barbiturates that there was no correlation between this effect of the drugs and their potencies as anaesthetics. This was an early indication that ganglionic blocking action may not be relevant to the anaesthetic effect of the barbiturates. It is interesting that although ganglionic transmission is cholinergic, inhibitory receptors for γ-aminobutyric acid (GABA) are present on ganglion cells. Pentobarbitone has been shown to potentiate or even to mimic GABA effects on a variety of preparations, including sympathetic ganglia, but whereas the peak effect occurs at around 8×10^{-5}M pentobarbitone (Bowery & Dray, 1978), a slightly higher concentration (10^{-4}M) has been measured in the blood on the recovery of consciousness (Richards, 1972). This observation therefore casts doubt on the role of the GABA-potentiation in pentobarbitone anaesthesia.

Anaesthetic effects on synaptic transmission having been established, the possible sites of action were examined in more detail. Somjen & Gill (1963) found reduced excitatory postsynaptic potentials (EPSPs) in ventral spinal roots and in single motor-neurones after peripheral stimulation, but no change in the resting membrane potential. However, they did not distinguish between the possible presynaptic and postsynaptic effects. Eccles, Schmidt & Willis (1963) reported increased and prolonged presynaptic

inhibition at low concentrations whereas inhibition was reduced by higher doses (reduced 50% by 30mg/kg). These authors were using cats and it should be noted that a dose of 30mg/kg induces surgical anaesthesia in this as in many other species.

Weakly (1969) was among the first to distinguish between presynaptic and postsynaptic effects: both pentobarbitone and thiopentone reduced the mean quantal content of an EPSP whilst leaving the amplitude of the unit potential unaltered. An anaesthetic-induced change in the input resistance of the motoneurone membrane was ruled out as a possible explanation and Weakly concluded that the barbiturate action was confined to inhibition of transmitter release from the presynaptic nerve terminal. These experiments also were carried out on cats, this time with sub-anaesthetic doses of pentobarbitone (10mg/kg).

In contrast, Thomson & Turkanis (1973) recorded an increase in the mean quantal content of EPPs at the neuro-muscular junction together with a reduction in the mean amplitude of miniature EPPs (MEPPs). The increase was the more remarkable because the external magnesium concentration was 10mM: magnesium and calcium are known to be antagonistic in their effects on transmitter release at the neuro-muscular junction (del Castillo & Engbaek, 1954). It is possible that this blocking effect of magnesium unmasked a second mechanism postulated for some anaesthetics; whereby they inhibit mitochondrial uptake of calcium, and so by increasing the intracellular concentration of free calcium, promote transmitter release (Krnjević, 1975). Under normal circumstances it may be that this is

masked by an effect of the anaesthetic to reduce the depolarisation-linked entry of calcium from the extracellular fluid. Such a reduction in the uptake of radio-labelled calcium by synaptosomes has been demonstrated (Blaustein & Ector, 1975). However, Thomson & Turkanis used phenobarbitone and a convulsant barbiturate, so their findings may relate more to excitatory than to anaesthetic actions of the barbiturates.

Haycock, Levy & Cotman (1977) showed that pentobarbitone (2×10^{-5}M to 2×10^{-4}M) reduced the calcium-dependent efflux of radio-labelled nor-adrenaline and GABA from synaptosomes. Their observation, that no such effect of pentobarbitone could be demonstrated when a calcium ionophore was incorporated in the system, localised the site of pentobarbitone's action to the depolarisation-linked entry of calcium. A similar reduction in GABA release was found when cortical slices were used. Waller & Richter (1980) extended this observation, showing that pentobarbitone (5×10^{-4}M) reduced only the calcium-dependent potassium-evoked release of endogenous GABA, glutamate, aspartate and acetylcholine from rat mid-brain slices, leaving the resting release unaltered. This reduced the likelihood that the results of experiments using radio-labelled exogenous transmitters were spurious, reflecting only the transmitters' uptake and release from neuro-glia (which also have a pronounced GABA uptake system). A comparable calcium-dependent potassium-evoked release of glycine has also been found, but so far only in the spinal cord.

Thomson & Turkanis' result was supported by that of Procter & Weakly (1976): both pentobarbitone and phenobarbitone in

concentrations up to 5 x 10^{-4}M progressively reduced the amplitudes of MEPPs and increased the quantal content of the EPP. The former effect was quantitatively the greater, especially with pentobarbitone, because the EPP amplitude was, if anything, reduced. A bathing solution with high magnesium (9-10mM) and low calcium (0.6-0.7mM) content was again used.

Meanwhile, Richards (1972, 1974) also had interpreted the results of his experiments with pentobarbitone in terms of a presynaptic effect. He measured evoked field potentials in slices of olfactory cortex, and found that pentobarbitone at 5 x 10^{-5}M to 6 x 10^{-4}M reduced the EPSP in a dose-dependent manner without affecting the compound action potential in the lateral olfactory tract. Since there was no change in the threshold for electrical stimulation of olfactory tract fibres nor for the synaptic excitation of post-synaptic cells, and post-tetanic potentiation and frequency potentiation were either unchanged or enhanced by 2-3 x 10^{-4}M pentobarbitone, he concluded that a reduced transmitter output from presynaptic terminals was likely.

Other studies have been concerned with inhibitory synapses. Recurrent inhibitory postsynaptic potentials (IPSPs) in cat motoneurones were prolonged by hexobarbitone (Larson & Major, 1970). This was not the result of a protracted Renshaw cell discharge. A range of anaesthetics has been shown to reduce the synaptic excitation of granule cells in the olfactory bulb, and to prolong the post-synaptic inhibition of mitral cells (Nicoll, 1972). A wide dose range of anaesthetics was effective. Reduced break-down of

GABA was ruled out as a possible explanation of the latter effect. Scholfield (1980) has recently shown that the inhibitory after-depolarisation recorded intracellularly from cells of the olfactory cortex was prolonged by all general anaesthetics he tested, including three barbiturates (among them pentobarbitone), but not by the local anaesthetic lignocaine. The effect was dose-related and was already considerable at concentrations that were still sub-anaesthetic. At higher concentrations (e.g. 2×10^{-4}M to 10^{-3}M pentobarbitone) he recorded depression of the early EPSP component of the response to stimulation of the lateral olfactory tract, and also a reduction in the input resistance of the neurones.

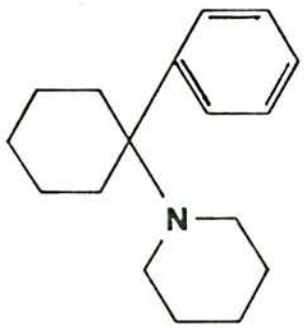
Barbiturates have thus already been shown to have more than one action relevant to synaptic transmission. Perhaps the best documented effects are those on the postsynaptic responses to GABA and glutamate. These will be considered in greater detail in Sections V (GABA) and VI (glutamate). Suffice it to say now that potentiation of GABA responses seems to occur in a variety of preparations, but is maximal at sub-anaesthetic doses. The depressant effects on glutamate responses occur with rather higher doses of barbiturates.

Phenylcyclohexylamines

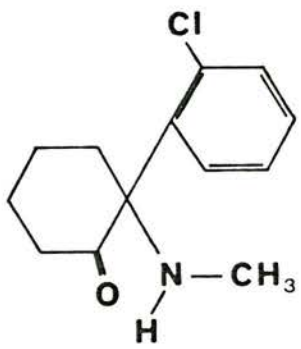
These compounds are derivatives of phencyclidine (Figure I-2), whose properties typify those of the group. Pharmacological effects of the parent compound (1-(1-phenylcyclohexyl)piperidine HCl) were described by Greifenstein et al (1958) in their report on the clinical trials, and by Chen et al (1959). Tests in mice and rats showed a quietening effect of low doses, but induction of a

Figure I-2

Structures of phencyclidine and its derivative ketamine



Phencyclidine



Ketamine

cataleptoid state or of general anaesthesia by high doses: this state progressed to convulsive seizures in some species. Non-toxic doses had few autonomic effects and, despite phencyclidine's having local analgesic activity approximately twice that of procaine, no depressant effect on skeletal muscle.

Thus it was apparent from the outset that here was a compound which, unlike the barbiturates or the volatile anaesthetics, produced a state of agitation or convulsions in high doses (0.5-1mg/kg); a lower dose (0.25mg/kg) allowed surgical incision and sometimes a complete operation. Amnesia occurred even though an element of consciousness remained during the operation. Muscle relaxation and cardiovascular depression were notable by their absence, but some respiratory stimulation was reported. However, disturbing psychotic side effects also occurred: in the clinical trials 10 out of 64 patients were unmanagable during the post-operative period, showing manic behaviour. Although the violent reactions could be controlled by opiates such as pethidine or morphine, phencyclidine was subsequently withdrawn from use in man. It has however been retained for use in non-human primates.

Ketamine, the representative drug used in this investigation, was developed during a search for a phencyclidine-like drug that was shorter acting and had no psychotic effects. First reports of its use in monkeys (McCarthy et al, 1965) and in man (Domino, Chodoff & Corssen, 1965) were favourable although some psychotic changes were recorded, usually vivid dreams or even hallucinations (Corssen & Domino, 1966). In appropriate dose, approximately 5 times that

of phencyclidine, ketamine produced similar analgesia, but the anaesthetic effect was of shorter and more controllable duration. A broad range of doses produced immobilisation, and subsequent tests showed that anaesthesia was induced in some species (monkeys, mice and pigeons) in which phencyclidine caused convulsions. The features of this type of anaesthesia which have led to its continued use are the good analgesia coupled with the maintenance of protective reflexes and the lack of depressant effects on the cardiovascular system: the latter makes it particularly suitable for use in poor-risk patients. However cardiovascular stimulation, thought to be central in origin, somtimes constitutes a contra-indication (White, Way & Trevor, 1982).

After its introduction into medical practice, ketamine was evaluated in several species of domestic animals including non-human primates (Beck & Dresner, 1972); cats (Beck, Coppock & Ott, 1971), pigs (Thurmon, Nelson & Christie, 1972) and dogs (Kaplan, 1972). More recently it has been used successfully in horses, in conjunction with xylazine premedication, and in birds. Excitatory side effects have been noted, particularly in pigs during recovery, but were controlled with thiopentone sodium or halothane. Excitement occurred more frequently in older animals and in animals that had been roughly handled. Convulsions reported in 3.4% of dogs were of the tonic-clonic type, all occurring between 2 and 7 minutes after the injection of ketamine and all responding to thiamylal. The routine premedication in Kaplan's study consisted of atropine, which partially countered the stimulation of salivation, acetyl promazine, to control hypertension, and thiamylal, which was found

to reduce muscle rigidity and make the results of anaesthetisation more predictable.

There were varying degrees of muscular hypertonus and purposeful movements occasionally occurred despite the level of anaesthesia's being such as to suppress motor responses to noxious stimuli. Interestingly, although interference with calcium binding or fluxes has been proposed to account for the biphasic effect of ketamine on the twitch response when muscle is stimulated directly (initial potentiation succeeded by a blockade), ketamine has been used safely in patients suffering from myopathies or known to be susceptible to malignant hyperthermia (White, Way & Trevor, 1982). The latter condition, which is triggered by certain anaesthetics and muscle relaxants, is thought to be related to an abnormality of calcium regulation in muscle cells (Hall, Lucke & Lister, 1980). Any general effect of ketamine on calcium metabolism is likely also to have repercussions on synaptic function.

Effects on the central nervous system (CNS) Much of the abundant literature concerning ketamine relates to attempts to define and explain the anaesthetic state produced. Corssen & Domino (1966) pointed out early on that although the reactivity of the CNS was altered, there was no true sensory blockade and the motor reflexes were not only intact but often hyperactive. The term "dissociative anaesthesia" was coined by Miyasaka & Domino (1968) to describe the state induced by ketamine. However, as Winters et al (1972) remarked, the former authors used doses of ketamine (1-16mg/kg) that were lower than those required to permit surgery (usually 20-40mg/kg). Winters suggested that ketamine be classified rather as

an excitatory agent capable of inducing a state of cataleptic anaesthesia; because surgical procedures could be performed only after doses that induced a cataleptic state that was accompanied by seizure activity in the hippocampus. This slow wave seizure activity, described also by Mori et al (1971) and Manohow, Maxwell & Winters (1972), was likened by Kayama & Iwama (1972) to the seizures of petit-mal epilepsy, and the two have since been shown to respond in a similar manner to the anti-epileptic drugs trimethadione and diphenyl hydantoin (Kayama, 1982). Kayama concluded however that the seizures could not, as had previously been supposed, be related to the hypnotic effects because whilst trimethadione inhibited seizure activity it did not alter the behavioural responses of cats to ketamine. It should be noted that Kayama, too, used sub-anaesthetic dose rates (2-6mg/kg i.v.). Seizure waves were confined to sub-cortical levels and Reder, Trapp & Troutman (1980), showing that ketamine (10-100mg/kg) protected two-day old White Leghorns against convulsions produced by pentylene tetrazole, concluded that ketamine has anti-convulsant activity in areas of the CNS which it does not stimulate. They also recorded that a dose of 100mg/kg, which was fully protective against convulsions, itself induced "bike-pedalling" activity in all birds tested.

There is general agreement that it is principally the association areas of the brain that are depressed by ketamine. It might therefore be predicted that in the simpler brain of the lamprey ammocoete (see p.38) ketamine would have little effect. However, ketamine has been used to induce anaesthesia in fish

(Oswald, 1978); although the dose required (130mg/kg) caused respiratory depression such that Oswald considered the drug contra-indicated. This dose is considerably greater than that used in mammalian species (20-40mg/kg) and it is possible that lower vertebrates are relatively insensitive to ketamine. It is interesting that much lower doses have been used clinically in man (1-5mg/kg i.v.; 7-13mg/kg i.m.): this, in view of the greater development of the association areas in the human brain, suggests that a different pharmacological action is important to the anaesthetic effect in different species. An alternative explanation may be that there are species differences in the permeability of the blood-brain barrier to ketamine.

Optical isomers of ketamine differ in their properties

A patent application for ketamine and related compounds, to be used as cataleptic agents in humans and animals, was filed in the U.S.A. in 1962 by Stevens on behalf of Parke Davis & Co.. Subsequently two optical isomers were separated and patented by Hudyma et al on behalf of Bristol-Myers Co.. Both isomers were anaesthetic and anti-convulsant, but pharmacological studies have since shown that the (+) isomer has greater potency (Ryder, Way & Trevor, 1978). At equi-analgesic doses in mice the (+) isomer caused less locomotor stimulation and had less hypnotic activity than did the (-) isomer, which suggests that the underlying mechanisms of these actions are distinct. Loss of the righting reflex was used as the index of hypnosis, and the "phenyl quinone writhing test" to assess the analgesic effect. These differences were quantitative: Meliska,

Greenberg & Trevor (1980) have since described qualitatively different effects of the isomers in behavioural tests in mice. Few studies have attempted to distinguish between the properties of the enantiomers, the majority using, as here, the racemic mixture.

Steroids

Interest in steroid anaesthesia began with the observation that intraperitoneal injection of several steroid hormones to mice and rats caused a reversible loss of consciousness (Selye, 1941). Subsequent investigation revealed pregnanedione as the most potent steroid tested, and as one without detectable hormonal effects (Selye, 1942). A derivative of pregnanedione, hydroxydione, was introduced into clinical practice in 1955, but was found to have certain disadvantages including a slow onset and long duration of action in comparison to thiopentone. More seriously, pain on injection and post-anaesthetic thrombosis were common, and the onset of side effects was delayed in the same way as was the anaesthetic effect.

The objective in developing steroid anaesthetics had been to find an intravenous induction agent which had a greater therapeutic index than the barbiturates and whose effect was terminated by some factor other than redistribution in the body. In many respects the steroids were promising: induction and recovery from anaesthesia were pleasant, surgical conditions were good (Sutton, 1972) and the action was terminated by detoxification in the liver. Attempts to resolve the problems of undesirable side effects and low water solubility were therefore continued.

Solubility had been reported by Selye (1942) to be an important factor limiting the anaesthetic effect of steroid hormones, and measures such as dissolving the steroid in a surface active agent, polyoxyethylated castor oil (Cremophor EL), have been used. Rapid induction and high potency were associated with a free 3α-OH group in the steroid molecule, and in a screening programme in which compounds were administered intravenously to mice, 3α-OH-5α-pregnane-11,20-dione (alphaxalone, Figure I-3) was the most potent tested. It was originally tested as an aqueous suspension (the British patent application in 1970 was for a sterile aqueous suspension or redispersed lyophilised solids) but was later marketed dissolved in Cremophor EL. Addition of a second steroid, alphadolone acetate (Figure I-3) further increased the solubility (by a factor of 3) whilst adding a lesser anaesthetic activity to the solution. A more concentrated solution could thus be prepared and the volume of an injection reduced by a corresponding amount. This product, known originally as CT 1341 and since marketed as Saffan for veterinary, and Althesin for medical use, has been used throughout the present study. Its composition is:

Alphaxalone	9mg/ml
Alphadolone acetate	3mg/ml
Cremophor E.L. (B.A.S.F.)	200mg/ml
Sodium chloride (AR)	2.5mg/ml

Water for Injection B.P. to 100%

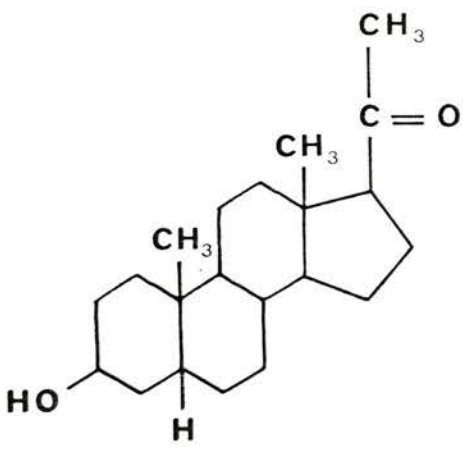
no antibacterial agent is included.

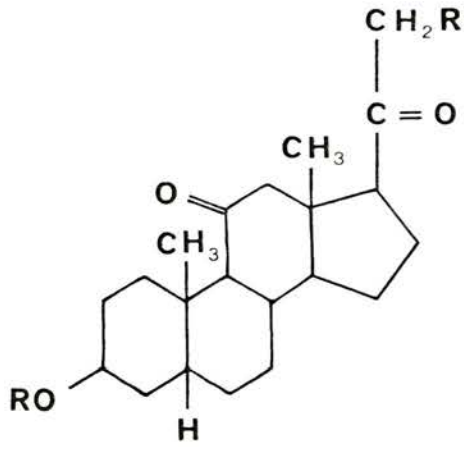
In the ensuing Sections this product is referred to as alphaxalone because concentrations are expressed in terms of this: the principal

Figure I-3

Structures of pregnane and its anaesthetic derivatives

Potent anaesthetics are produced by making simple substitutions on the pregnane molecule (left). In alphaxalone (right) "R" = H. In other anaesthetic derivatives "R" may be H, OH or an ester (usually succinate or phosphate).


CH3
C = O
CH3
CH3
HO
H


CH2R
C = O
CH3
O
CH3
RO
H

active constituent.

There has been a continued search for a water-soluble anaesthetic steroid that is also acceptable in other respects: one promising contender, minaxolone, has been withdrawn pending long term toxicity studies. Other side effects remain a problem and adverse reactions occur rather more frequently with Althesin than with the barbiturates, sometimes leading to cardiovascular collapse. These reactions appear to be of two types: one, involving Complement, occurring on first exposure in subjects who frequently have previously shown atopic phenomena, and the other, a hypersensitivity reaction, occurring on repeated exposures (e.g. Radford, Lockyer & Simpson, 1982).

Child et al (1971) reported on anaesthetic tests using CT 1341 in a variety of mammalian species, and the drug then passed into clinical trials. Early clinical experiences were described by Campbell et al (1971) and Clarke et al (1971). Both groups reported muscle movements or tremors in some patients, excitatory effects that were dose-related. However, whereas the latter found the incidence of excitement to be 5% at 30 μl/kg, 50% at 100 μl/kg and 75% at 200 μl/kg; the former claimed that involuntary movements occurred more frequently at their lower dose rate (100 μl/kg as opposed to 150 μl/kg) and when the patient was stimulated. Comparing CT 1341 with other available induction agents, Clarke et al found the incidence of excitatory effects second only to that after methohexitone administration. These effects occurred soon after injection and subsided spontaneously.

Subsequent studies evaluated the new drug for use in domestic animals including cats (Child et al, 1972; Evans, Aspinall & Hendy, 1972), horses, sheep and pigs (Hall, 1972). Apart from hyperaemia, there was said to be a low incidence of side effects in cats, but those reported included muscle twitching and occasional emergence excitement. Excitement constituted a more serious problem in horses when paddling and galloping movements alternated with mild trembling and contractures of isolated muscle groups during the recovery period. These effects, reduced by acetyl promazine and almost abolished by xylazine, are of particular interest in relation to the lamprey study, as are the occasional excitatory reactions reported in other species. Hall did not comment on excitement in pigs or sheep, but reported a low incidence in cats (3 out of 71). The low water solubility was a particular problem in the larger species because of the volume of injection necessitated. Histamine release caused by Cremophor E.L. rendered the product useless in dogs unless an anti-histamine drug was incorporated in the premedication.

Modifications of the alphaxalone molecule, such as the introduction of substituents and double bonds, alter its anaesthetic activity. Rearrangement of the molecule as in Δ^{16} -alphaxalone, can lead to the loss of anaesthetic activity. Richards & Hesketh (1975) showed that Δ^{16} -alphaxalone (5×10^{-5}M) depressed the population EPSP measured in the olfactory cortex by only 10-15%, whereas the depression due to alphaxalone was 30-60%. In contrast, both compounds reduced the depolarisation-secretion coupling as assessed by measurement of MEPPs at the mouse neuro-muscular

junction (Pennefather, Puil & Quastel, 1980). Lawrence & Gill (1975) found that the β- isomer both was inactive as an anaesthetic and produced less disordering of phospholipid/cholesterol bilayers; perhaps because, being a planar structure, it could more readily be incorporated into the membrane without causing disruption of its structure. Comparison of the properties of the various isomers should assist the identification of effects important to anaesthesia.

Effects on the CNS The neuropharmacology of alphaxalone is not well documented. However, the effects on the EEG seem to be excitatory as are those of γ-OH butyrate (Bimar & Lepouleuf, 1973; Timms, 1976). Alphaxalone has been reported to precipitate epileptic convulsions in susceptible individuals (Uppington, 1973), an action also reported for hydroxydione and methohexitone, but not for thiopentone. Anti-convulsant activity of alphaxalone has also been demonstrated: against generalised convulsions induced by pentylene tetrazole (Bimar & Lepouleuf, 1973) or exposure to hyperbaric oxygen; and against focal seizures produced by local application of penicillin (de Riu, Susini & Ruju, 1982). Cases of benzodiazepine-resistant status epilepticus have been successfully treated with alphaxalone. The apparent conflict may stem from the doses used: de Riu et al gave a dose of 0.9mg/kg alphaxalone (0.1ml/kg Althesin) to rabbits, whereas the anaesthetic induction dose in this species is about 18mg/kg. Also, the focal convulsions were induced by the application of penicillin to the pia of rabbits already anaesthetised with ether. The only major discrepancy Gyermek & Soyka (1975) reported between the central actions of

hydroxydione and those of the barbiturates was that the latter depressed monosynaptic reflexes unaffected by the steroid and caused a more complete block of polysynaptic reflexes. A direct comparison between alphaxalone and barbiturates was not made but the EEG changes were similar: spindles appeared and progressed to hypersynchrony. The main effect of alphaxalone was on the sensory and limbic systems; evoked potentials in the reticular formation were unchanged or depressed.

In contrast to the 5β- steroids, alphaxalone does not seem to cause hyperaesthesia. Smaje (1976) and Richards & Smaje (1976), recording spike activity extracellularly, have shown that 5×10^{-5} M alphaxalone reduces the sensitivity of guinea-pig prepyriform cortical neurones to both acetylcholine and glutamate.

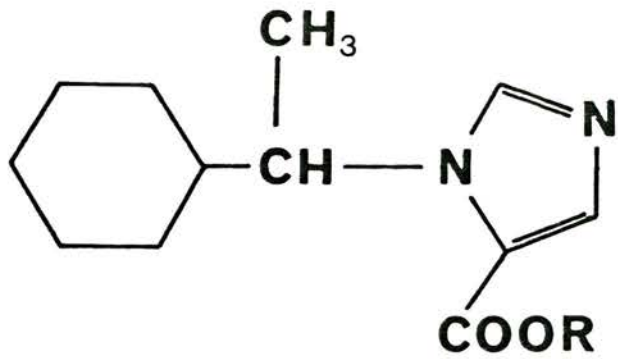
Imidazole derivatives

Following reports of the use of the imidazole derivative "methoxymol" for the anaesthesia of birds (Marsboom, Mortelmans & Vercruysse, 1965; Cooper, 1970), Janssen et al (1971) published the results of animal tests with a new derivative, etomidate. These, and subsequent experimental studies and clinical trials, suggested that here was a successful short-acting hypnotic with a rapid onset of action and minimal effects on the circulation. Etomidate was first used in man in 1972 under ECG and EEG control. It is the ethyl ester of 1-(1-phenylethyl)-1H-imidazole-5-carboxylic acid (Figure I-4), and is generally marketed as the more soluble hydrochloride. Detoxification is by hydrolysis probably mainly in the liver (Ghoneim & van Hamme, 1979), and the kidney is the only

Figure I-4

Structures of anaesthetic imidazole derivatives

"R" represents a methyl group in metomidate, and an ethyl group in etomidate.


CH3
CH
N
N
COOR

important route of excretion (Meuldermans & Heykants, 1976). All metabolic products were found to be pharmacologically inert.

Doenicke et al (1982) administered etomidate intravenously by bolus injection and infusion. Each injection caused a steep rise in the plasma concentration and a moderate deepening of hypnosis, whereas during infusion there was no such change in depth and only a moderate increase in plasma concentration. Rapid exchange of etomidate between the blood and cerebro-spinal fluid (CSF) was correlated with the short hypnotic activity. Schüttler et al (1980) using an infusion that maintained the plasma concentration at about 0.5 μg/ml found that the concentration on recovery was about 0.3 μg/ml, whereas Doenicke et al reported that recovery occurred at 0.13 μg/ml.

The methyl ester also has hypnotic action (Godefroi & Platje, 1972) and was introduced into veterinary practice at about the same time. Reports appeared concerning its use in pigs (Callear & van Gestel, 1973; Symoens & Gestel, 1973, 1974), horses (Hillidge, Lees & Serrano, 1973) and cats (Erhardt et al, 1978); since when it has been used clinically, mainly in pigs. Clinical reports on the two drugs were uniformly favourable although a relatively high incidence of non-purposive muscle movements and of myoclonia was mentioned by some authors (e.g. Kay, 1976; Holdcroft et al, 1976). The latter, having compared the incidence of various side effects of etomidate anaesthesia in man after four premedication routines, reported that in many patients the excitatory phenomena evoked were reflex and that opiate premedication made induction acceptable in more than 90% of patients. Dundee et al (1961) have

reported that pain can aggravate the excitatory muscle reactions seen during methohexitone anaesthesia, and an equivalent effect may underlie Holdcroft's findings. Studies of the use of metomidate in domestic animals have usually incorporated a premedicant: the butyrophenone azaperone in work on pigs, or the opiate fentanyl in dogs and cats. Erhardt et al (1978) reported cramps and ataxia in about 50% of cats during induction and recovery when fentanyl was the premedicant; but since this ataxia was reduced by treatment with the opiate antagonist Levallorphan, it probably arose from the actions of fentanyl. A low incidence of tremors (4%) and myoclonia (0.8%) was reported when azaperone was used as the premedicant in pigs (Symoens & van Gestel, 1973).

Effects on the CNS Both etomidate and metomidate occur as two optical isomers. Those of etomidate have been studied separately and only the (+) isomer was found to be hypnotic when injected into the cerebral ventricles (Hill & Taberner, 1975). The (-) isomer failed to cause loss of the righting reflex in mice in doses of up to 500 μg, whereas the ED_{100} for a 10min loss of the reflex was 67 μg for the (+) isomer.

Available reports suggest a GABA-mimetic action of (+)-etomidate in several preparations: etomidate-induced depolarisation was depressed by known GABA antagonists (Evans & Hill, 1978). The previous report (Hill & Taberner, 1975) of an effect of (+)-etomidate to depress the firing of single neurones in the medulla was open to several interpretations and in any case occurred at supra-anaesthetic concentrations (5×10^{-3} M, whereas concentrations

in the range 5 x 10^{-6} M to 3 x 10^{-5} M have been measured during etomidate hypnosis). *In vitro* tests using rat brain tissue showed that neither isomer depressed the mitochondria when applied at hypnotic concentrations, nor did they affect the active uptake of radio-labelled GABA or glutamate. EEG changes occurring during metomidate and etomidate-hypnosis have been likened to those effected by the anaesthetic barbiturates (Doenicke *et al*, 1982).

The Preparation

Although this dissertation is the report of a pharmacological study of selected anaesthetic agents and is therefore principally concerned with the drugs, brief comment on the biology of the lamprey and on the reasons for the selection of the reticulo-spinal cell preparation is appropriate.

General

Lampreys, of which several species exist, together with hagfishes are the sole extant Ostracoderms, palaeozoic jawless fishes. Grouped as the Cyclostomes, they are eel-like aquatic chordates with a very primitive CNS. Because of their transitional evolutionary position, lampreys have been studied extensively by comparative neuro-anatomists so that a wealth of literature is available. However, some (e.g. Nieuwenhuys, 1977) dispute that they can properly be regarded as the phylogenetic ancestors of the higher vertebrates, arguing that they have developed independently from the hypothetical common ancestral craniate. Notwithstanding this caution, it is recognised that the lamprey brainstem retains many primitive features and contains the rudiments of structures, such as the hippocampal cortex, which are developed to a far greater extent and assume a functionally important role only in the higher vertebrates.

Taking a linear view of the evolutionary process, Karamyan (1972, 1975) placed the cyclostomes at Stage II in the development of the CNS. In cyclostomes, integrative functions are performed by the mesencephalic/bulbar apparatus; and although afferents

transmitting information of different modalities have distinct distributions within the brain, individual central neurones are polymodal and the organization of the afferent system remains diffuse. Diffuse organization is regarded as a primitive feature. By contrast, the next stage (Stage III), which includes the fishes, is marked by the development of the cerebellum, this together with the mesencephalic nerve formations becoming the main apparatus for the integration of afferent information.

Several recent reviews of the neuro-biology of lampreys are available and the following account draws on those of Shapovalov (1975), Nieuwenhuys (1977), Hardisty (1979) and Rovainen (1979).

Reticulo-spinal cells

The bulbar reticulo-spinal cells that were used in this study form part of the somato-motor system. The reticulo-spinal projection is believed to be the most fundamental - phylogenetically the oldest - direct line of supra-spinal motor control. It is present in all classes of vertebrates from the cyclostomes to the mammals, and is not subject to much interspecific variation, occupying a constant position in the brain and spinal cord. Its antiquity and persistence testify to its continuing importance, although the precise role of this system has altered during the evolution of the CNS. In mammals rapid motor signals are carried by the pyramidal tracts and α-motoneurones, but there is also increased dependence of the spinal motor centres on higher centres in the brain: the γ-cells and Renshaw cells are influenced by supraspinal structures.

The bulbar cells are among the most prominent cells in the lamprey brainstem and can readily be seen with the aid of a dissecting microscope (x 40). Giant cells occurring bilaterally in pairs were first described in the 19th Century (Müller, 1840) and were found to be constant in position between specimens. These Muller cells have been subjected to many subsequent studies (e.g. Johnston, 1902; Tretjakoff, 1909; Saito, 1928; Stefanelli, 1933) as have the giant axons present in the spinal cord. There was however no consistent definition of a Müller cell in the classical literature. For the purposes of this dissertation the classification of Rovainen (1967) will be used: he defined Muller cells as the cells of origin of the 8 pairs of uncrossed giant axons in the medial longitudinal fasciculus of the spinal cord. These cells are located in three brain regions:

the mesencephalon (cells M1 - 3);

the isthmus separating the 3rd and 4th ventricles (I1);

the rhombencephalic basal plate (B1 - 4).

It is cells of the latter group that have been used for this study, the bulbar cells lying near the midline in the centre of the medulla immediately under the ependymal layer (Tretjakoff, 1909). Nieuwenhuys (1972), in his slightly different classification, termed this region the Nucleus reticularis rhombencephali medius. Smaller reticulo-spinal cells also occur in this location but are less readily seen and were not used in this investigation: the column of reticulo-spinal cells is in fact continuous from the tegmentum to the (lower order) primary somatomotor cells of the caudal hindbrain (Addens, 1933).

Afferent connections: Tretjakoff (1909), using a Golgi staining method, was the first to demonstrate the dendrites of the Müller cells. More recently, Martin (1977, 1979b) used an intracellular injection technique with the dye procion yellow to investigate the ramifications of the dendritic trees of the Müller and Mauthner cells. Figure I-5 shows six bulbar cells so stained. The bulbar cells had ovoid somata with the long axis oriented transversely, and their dendrites extended laterad, ventrad and caudad but did not cross the mid-line. Comparing the dendritic trees with those of mammalian reticulo-spinal neurones, Martin pointed out that both were primarily distributed in a transverse plane, and both intermingled freely with passing (descending and ascending) fibres.

As for the source and type of information carried by the afferent fibres, Veselkin (1966) recorded the activation of large axons in the medulla and spinal cord following optic nerve stimulation; whereas Rovainen (1967) recorded, in nearly all reticular neurones tested, some combination of EPSPs and IPSPs in response to electrical stimulation of the optic, trigeminal, vestibular and lateral line nerves. There was no consistent pattern of response, nor was there any response to olfactory nerve stimulation. Rovainen made no attempt to mimic the natural stimuli, but deduced from the response latencies and the anatomical relations of the Müller cells that the sensory paths were polysynaptic, possibly (Nieuwenhuys, 1977) being relayed in the tectum. Nieuwenhuys considered that the existence of a spino-reticular projection was likely, but none had then been described.

Afferent synapses have also been described throughout the

Figure I-5

Bulbar reticulo-spinal cells (Müller cells) stained with procion yellow.

Orientation as in Figure II-3: long axis vertical with rostral end at the top. Magnification x180

(R.J. Martin, reproduced with permission)

length of the giant axons. These distributed synapses are by their location, adjacent to the efferent synapses with segmental motoneurones, admirably placed to modify the output to individual motoneurones by local polarisation effects. Sub-threshold EPSPs have been recorded (Rovainen, 1967), temporal or spatial summation being necessary for impulse trains to be generated in the motoneurones. Electrophysiological evidence for the existence of the distributed synapses was presented by Rovainen (1967), and involved simultaneous recordings from the cell body and axon whilst the ipsilateral trigeminal nerve was stimulated.

Efferent connexions: The principal efferent channel for impulses from the soma of a Müller cell is the giant axon. Müller axons run in the ipsilateral cord and are thought to act as pre-motoneurones in that they synapse directly with the segmental motoneurones (Rovainen, 1974). Morphologically these are mixed synapses, with characteristics both of electrical and of chemical junctions. Before the axon widens to continue as a giant axon, there is a narrow section in the brainstem which, according to Martin (1979b) could permit the integration of synaptic currents at the axon hillock.

In contrast to the abundant axon collaterals of mammalian reticulo-spinal cells, the giant axons of the lamprey Müller cells are generally unbranched (Rovainen, 1967). However, a detailed study of the brain and spinal cord of a single adult sea lamprey by serial sections revealed not only some giant axons that migrated from the ventral columns of the cord as far as the dorso-lateral

column, but also that these deviant axons were mostly branched. None however was observed to cross the mid-line (Rovainen et al, 1973).

The size of the Müller axons, and their action as the functional terminals of the cells, making synapses directly with the segmental motoneurones at all levels of the cord, suggest that this is a channel for the rapid transfer of information that requires little peripheral modification (see Rushton, 1951). The cell bodies of the primary motoneurones are located immediately lateral to the giant axons with dendritic trees that are confined to the ipsilateral side. Both "fast" and "slow" primary motoneurones occur.

Buchanan & Cohen (1982) recorded EPSPs from almost all slow muscle fibres tested when swimming was induced by the application of D-glutamate to the spinal cord. Action potentials were found in twitch fibres only during high intensities of bursting activity in ventral spinal roots. However, they also found that repetitive stimulation of Müller cells during such induced swimming evoked an increase in "burst" activity in the ventral roots which was associated with an increase or decrease in the frequency of occurrence of bursts. Increased activity in Müller cells should therefore result in an increased activity of twitch fibres. These considerations are consistent with Rovainen's finding (1967) that movements elicited by intracellular stimulation of bulbar axons included undulations or rapid waves of contraction spreading caudad, and that with high frequency stimulation (100/sec), twitches of the whole ipsilateral body occurred. Rovainen (1967) did not

distinguish between the movements elicited by stimulation of the various bulbar axons. However, he subsequently reported that B3 and B4 make excitatory synapses with ipsilateral myotomal motoneurones and lateral interneurones whereas B2 excites only the latter. The information concerning the efferent connexions is still fragmentary but implies that B3 or B4 could activate undulatory swimming movements.

In relation to the movements elicited by stimulation of the bulbar cells, it is interesting that Selzer (1978) found that swimming movements were restored several weeks after complete spinal cord transection in lampreys, but only at a time when some limited regeneration of the reticulo-spinal axons could be demonstrated. Histological and physiological evidence indicated that there was no regeneration of long axon tracts: 12 out of 18 axons regenerated, were branched and had migrated from their normal path. Selzer suggested that new synapses were formed between the axon sprouts and proprio-spinal interneurones, and that it was through these contacts that descending volleys initiated rostral to the lesion could activate giant interneurones. The implication of Selzer's work was that some signal from higher centres is required to initiate swimming.

Transmitter systems: Both excitatory and inhibitory afferent inputs to the bulbar Müller cells have been described, but the natural transmitter has not been positively identified at any synapse. A wide range of putative neurotransmitters is now recognised and several of these have been applied to the cells in an attempt to

identify likely candidates for the role of natural transmitter. The cells are known to respond to exogenous GABA and glycine (Martin, 1978; Matthews & Wickelgren, 1979a), glutamate and aspartate (Matthews & Wickelgren, 1979b). Of these Matthews & Wickelgren favoured glycine as the inhibitory, and glutamate as the excitatory transmitter. These responses are considered in greater detail in Sections V & VI.

Transmitters tested and found to be without detectable effect included acetylcholine, carbamylcholine, noradrenaline, dopamine, histamine and 5-HT (Matthews & Wickelgren, 1979a). The observation with acetylcholine was consistent with the earlier finding of Wachtler (1974) that acetylcholinesterase activity, although present elsewhere in the lamprey brainstem, was absent from the Müller cells and Müller fibres.

The lamprey as a model for the study of the effects of anaesthetics

> ...pour comprendre cette action ainsi interprétée, rappelons que le chloroforme n'agit pas uniquement sur les éléments nerveux: loin de là, cet agent porte en réalité son action sur tous les tissus; il atteint chaque élément à son heure, suivant la susceptibilité ... l'effet se manifeste sur les autres tissus, après qu'il s'est déjà manifesté sur le tissu nerveux, le plus délicat de tous ... l'anesthésique n'est donc pas un poison spécial du système nerveux; il anesthésie tous les éléments, tous les tissus en engourdissant, en arrêtant momentarément leur irritabilité nutritive...
>
> Bernard (1875)

Bernard sought to justify the use of non-nervous tissues in experimental studies of anaesthesia. In the light of this the lamprey reticulo-spinal cells seem admirably suited for such a

study: not only do they have counterparts in the mammalian brain, but they respond to the same neuro-transmitters in an ostensibly similar manner. However, Bernard claimed too much. It is true that anaesthetics depress tissues other than the brain, but it is not necessarily true that all effects contributing to an anaesthetic state are produced in the same way or are manifestations of a single non-specific action.

Lamprey ammocoetes were readily available in local waters and could be maintained satisfactorily for long periods. The lamprey brainstem has several features that make it a suitable preparation in which to study the neuropharmacology of anaesthetic drugs. It combines one of the virtues of invertebrate preparations - accessibility - with the CNS organisation of a vertebrate. Experiments could be carried out on identified cells which yet retained many of their normal relationships. Intracellular recording enabled changes in input resistance and membrane potential to be measured. This was preferable to the use of indirect measures of cell responses, such as changes in spike frequency. The bulbar reticulo-spinal cells lie close to the surface of the 4th ventricle, covered only by a single layer of ependymal cells; thus the diffusion path for drugs applied in the perfusing Ringer is short (Martin, 1979a,b). By applying drugs directly to the cells the complicating influence of indirect circulatory effects was avoided. In the lamprey brain, blood vessels are confined to the periphery and so neither obscure nor impede access to the cells. Visualisation is also aided by the absence of myelin.

Most of the experiments described in this dissertation

(Sections V & VI) were concerned solely with postsynaptic effects. Furthermore, they have taken no account of processes subsequent to the postsynaptic potential: no measurement of the impulse-generating process has been made. These experiments represent an advance inasmuch as anaesthetic effects on identified brain cells in situ have been examined. These are cells some of whose connexions are known, so prediction of the consequences of observed effects in the living animal has a solid foundation. As already described, the cells are thought to constitute a major motor outflow to the somatic musculature of the lamprey. The equivalent cells in mammals also have a motor function (Shapovalov, 1975). Any stimulation of the neurones by anaesthetics, effected either directly or via the removal of inhibitory influences, should therefore elicit movements.

The results presented add to the growing body of data which indicates distinct actions for different anaesthetics, but whether any or all of them relate to mechanisms of anaesthesia must be a matter for conjecture. The lamprey is a simple animal probably not consciously aware of its surroundings; the mammalian reticulo-spinal tract in any case is not concerned with mechanisms of consciousness, and no role for the amino acid transmitters in such mechanisms has been demonstrated. The techniques employed in this study involve application of putative transmitters in the general vicinity of the cell; these substances may not be identical to the natural transmitters and may not necessarily act on the same receptors that are involved in normal synaptic transmission. Although it cannot be certain how far results obtained using the lamprey preparation are

applicable to anaesthetic-induced unconsciousness in mammals, it seems likely that they are relevant to the problem of voluntary muscle movements which may occur during anaesthesia, and probably to other brain functions which involve these same transmitters: the properties of amino acid receptors are remarkably similar in different preparations.

SECTION II

Materials and Methods

Experimental Animals

Lamprey ammocoetes were obtained locally from tributaries of the Tweed. They were most readily caught in shallow areas of the stream where the bed was mud or sand and the water was slow moving. Initially electric fishing tackle belonging to the Tweed Commissioners was used at the mouth of the Eden water near Kelso (Grid reference: NT 766 376). Latterly, Departmental equipment became availableand was used in the Eddleston water, between Eddleston and Peebles (Grid reference: NT 238 454). This equipment (Safari 100 Man Pack Electro Fisher: Marine Electrics Ltd. Donegal) was designed with a landing net as the anode and, as the cathode, a metal plate which could be trailed on the stream bed. It was powered by two 12 volt batteries and gave out pulsed d.c. at frequencies up to 400 Hz. Unidirectional pulsating current causes fish to swim towards the cathode; if sufficiently strong, it paralyses them (see Madden & Houston, 1976). The effect on lampreys was to attract ammocoetes from their burrows in the stream bed and then to paralyse them. Lampreys, unlike small fish in the vicinity, were not always totally immobilised but their movements were so sluggish that they could readily be netted or picked out by hand.

The ammocoetes caught were of two species: Lampetra fluviatilis (river lamprey) and Lampetra planeri (brook lamprey). The species differ slightly in appearance but no distinction relevant to this investigation has been reported, either in the organization of the brainstem or in the neuropharmacology of the reticulo-spinal system. The responses of the bulbar Müller cells are also similar to those of cells in the sea lamprey

(Petromyzon marinus) on which many previous experimental studies have been performed (e.g. Rovainen, 1967; Wickelgren, 1977; Matthews & Wickelgren, 1979a,b).

In the laboratory the ammocoetes were stored in plastic tanks measuring 44 x 38 x 18cm. The tanks were periodically replenished with fresh stream water and were aerated using standard aquarium pumps (Whisper 400: Interpet). Supplementary feeding proved unnecessary when the tanks were provided with washed sand or grit from the stream bed and the temperature was maintained at 4-6°C. The sand enabled the ammocoetes to make burrows and also provided a limited supply of nutrient material; additional feeding would have increased the organic material present and so have added to the oxygen demand. Tanks were always thoroughly cleaned before a new batch of animals was introduced.

The Dissection

Ammocoetes 8-15cm long were anaesthetised with neutralised tricaine methane sulphonate (10^{-4}g/l) and then transected caudal to the gills. The rostal portion was divided in the ventral midline and, after tissues covering the ventral notochord had been removed, was pinned out on Sylgard (an elastomeric silicone resin) in the experimental chamber, dorsal surface uppermost. A longitudinal mid-line incision exposed the brainstem. Removal of the choroid plexus then exposed the bulbar reticulo-spinal cells, near the centre of the 4th ventricle clustered on either side of the midline (Figures II-1 to II-3). The preparation was continuously perfused with Ringer solution at 6ml/min.

Figure II-1

Two lamprey ammocoetes

(diameter of petri dish 90mm)

Figure II-2

Dorsal view of brainstem

Orientation transverse with rostral end to the left.

Tissues covering brainstem divided in midline and pinned back.

Choroid plexus removed from 3rd and 4th ventricles, and isthmus divided.

Magnification x7

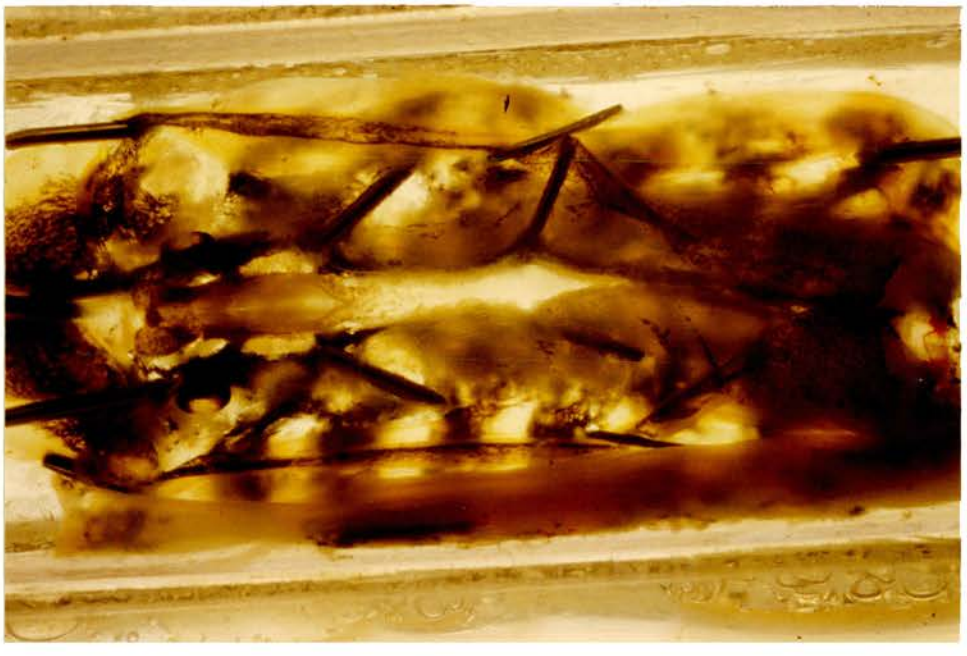
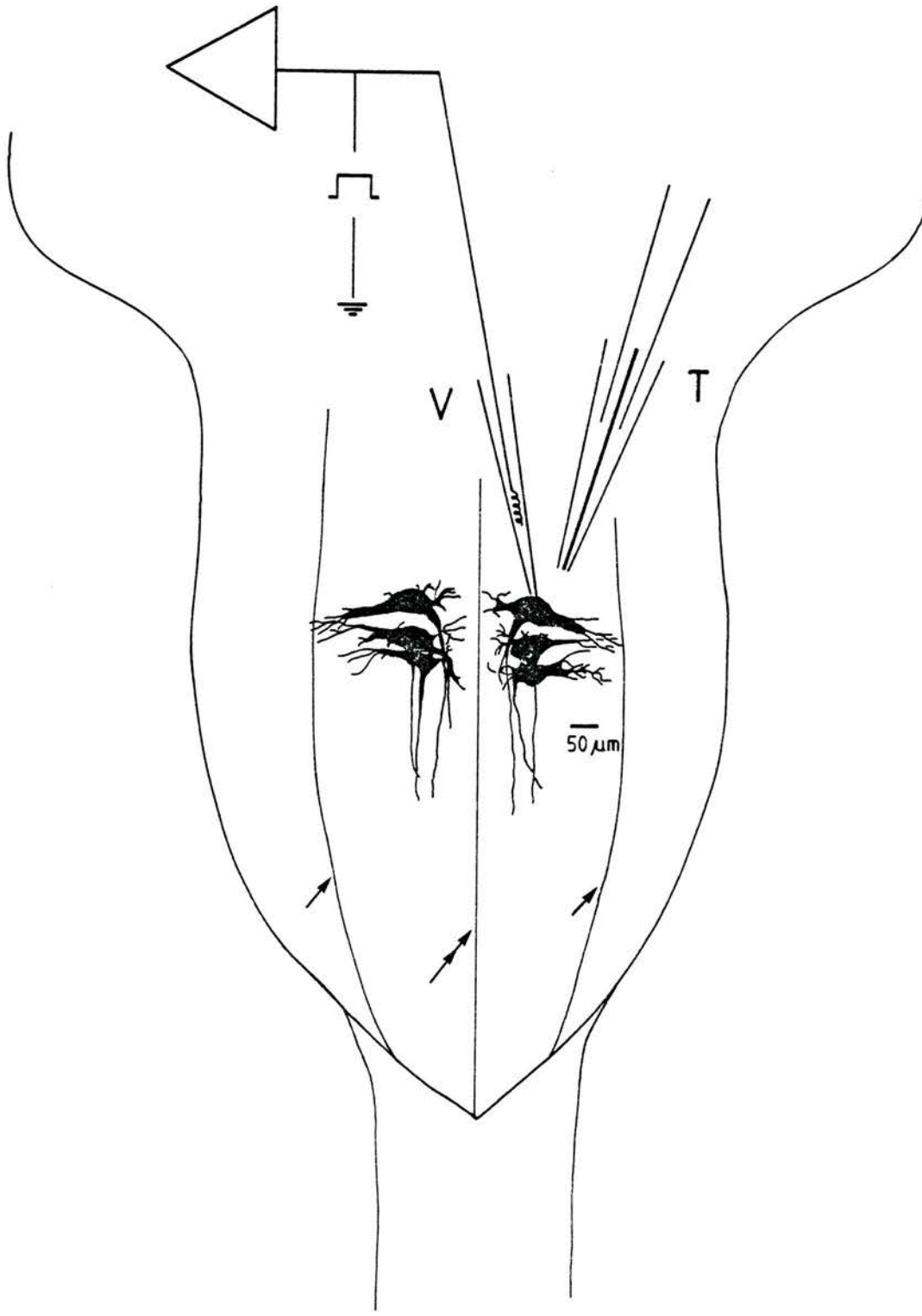


Figure II-3

Diagram of the preparation showing the location of micropipettes.

Bulbar reticulo-spinal cells (Müller cells) lie in the floor of the 4th ventricle between the sulcus limitans of His (arrows) and the median longitudinal sulcus (double arrow). Positions of recording (V) and iontophoresis (T) electrodes are indicated.


V
T
50 µm

Experimental Arrangement

The experimental chamber was constructed from perspex and contained a central rectangular well with a drainage hole at one end. After a layer of Sylgard 184 had been placed at the bottom the liquid capacity of the well was 1ml. Beneath and around the sides of the central well was an outer compartment through which could be circulated cold fluid (see Figure II-4). The chamber was mounted on a perspex base which was screwed to three rods and thence to the cast iron base plate. This plate, to which were bolted also the micro-manipulator mountings, rested on dense foam blocks. The blocks, which acted as shock absorbers, rested on concrete paving slabs placed across a rigid metal trestle. Micro-manipulators (Zeiss) were used to hold the recording electrodes and those used for the iontophoretic application of transmitters. The chamber was illuminated from below and the preparation viewed through a dissecting microscope (Olympus zoom microscope). Müller cells were easily seen using the low power of the microscope. The microscope was mounted separately so as to avoid unnecessary jolting of the table.

Cooling was achieved by a flow heater (FH 15: Grant Instruments (Cambridge) Ltd.) used in conjunction with a refrigerated flow cooler (FC 15) and bypass system (BS 15). The system was filled with proprietary anti-freeze, and a continuous record of temperature in the well was made by a thermistor probe connected to the pen recorder. This temperature was controlled to ± 0.5°C, and its relation to the temperature set at the circulator is indicated in Figure II-5. A high degree of control was necessary because the

Figure II-4

Diagram to show experimental arrangement

Dark shading in experimental chamber represents layer of Sylgard.

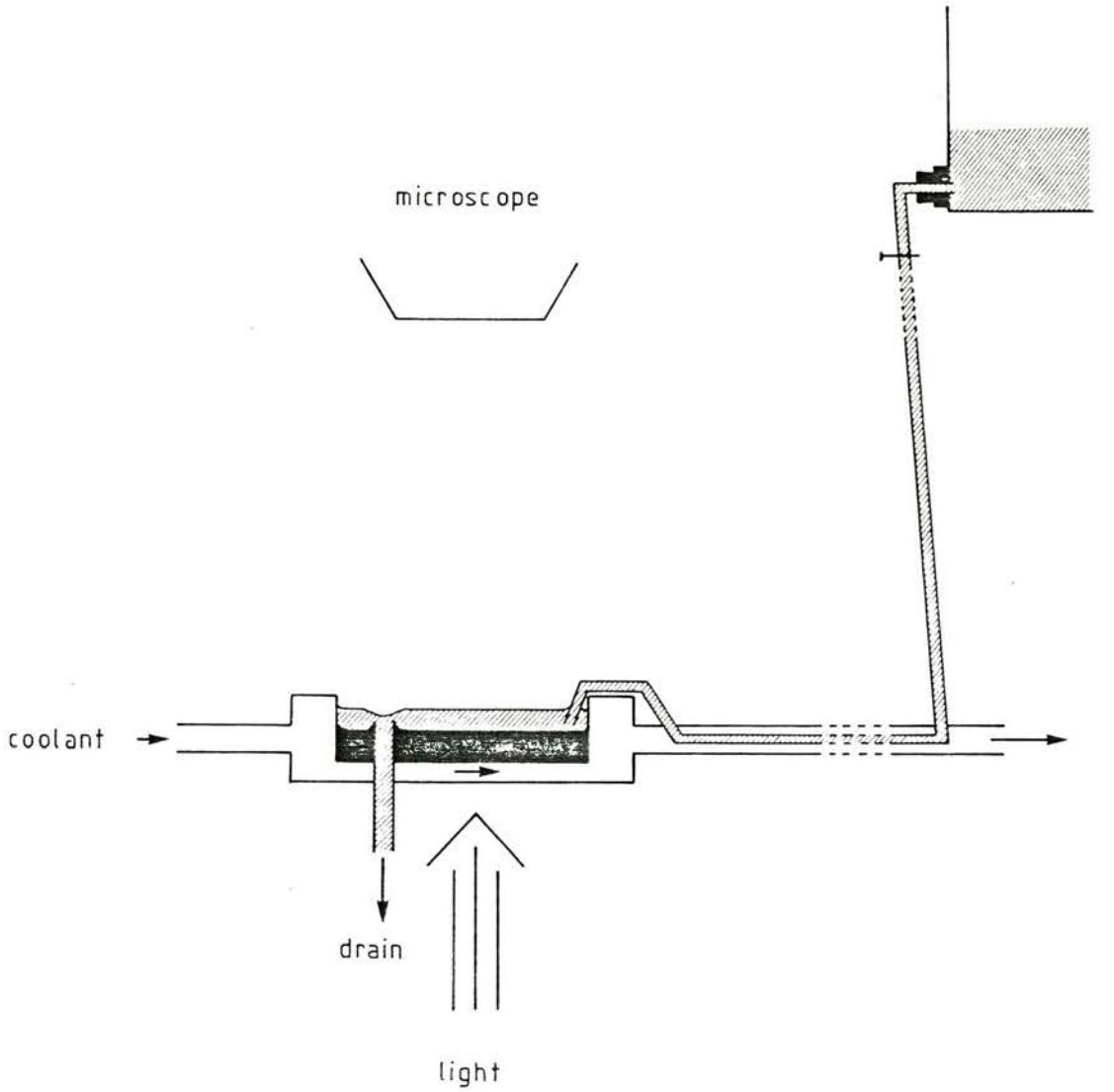
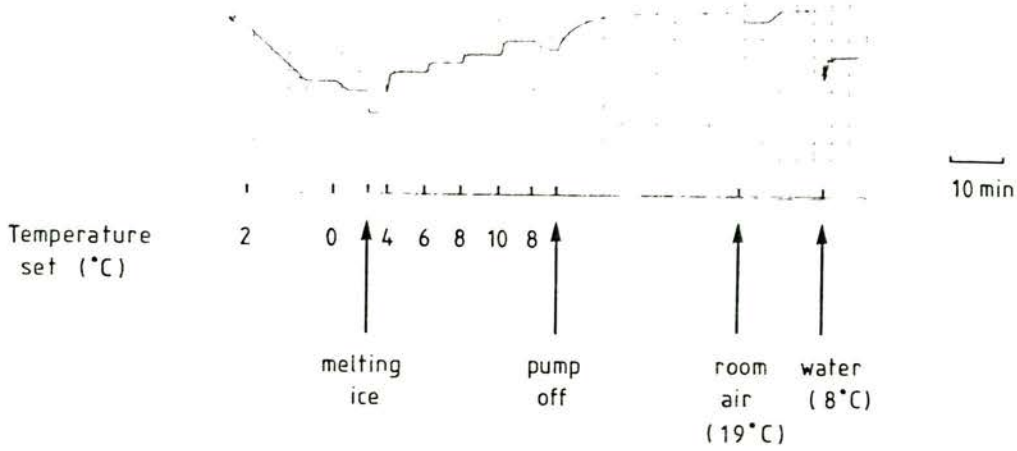

microscope
coolant
drain
light

Figure II-5

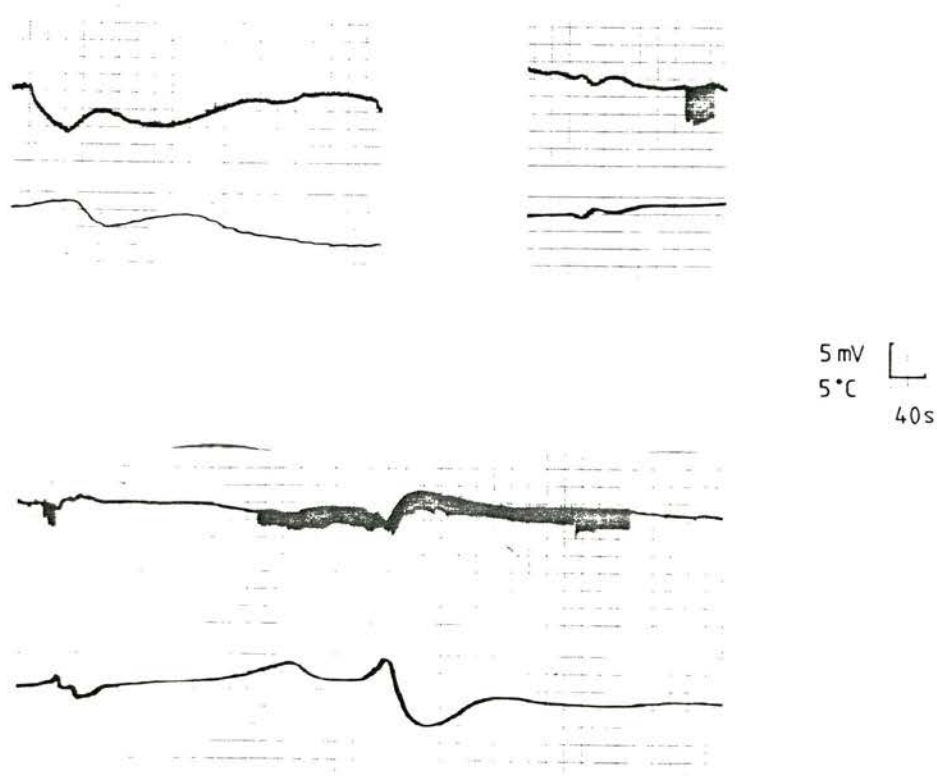
A: Relation of temperature in experimental chamber to that set at flow cooler: a continuous record with thermistor movements and alterations in temperature settings at times indicated.

B: Variations of membrane potential (upper traces) with temperature in chamber (lower traces).
Direction of changes: hyperpolarisation and decrease in temperature downwards. Downward transient deflections of the membrane potential are in response to injected current pulses.

A


10 min
Temperature
set (°C)
2
0
4
6
8
10
8
melting
ice
pump
off
room
air
(19°C)
water
(8°C)

B


5 mV
5°C
40s

resting membrane potential of the bulbar Müller cells showed marked temperature sensitivity (see Figure II-5). The thermistor was calibrated against a mercury thermometer, using as fixed points: melting ice, and cold tap water (16°C).

Perfusion System

A continuous flow of Ringer through the chamber was provided in early experiments by a peristaltic pump (Ismatec). The pump was later abandoned in favour of a system of gravity feed, which allowed solutions to be changed with minimal disruption of the flow and with more precise timing. This change also removed one source of electrical interference. Two aspirator jars were clamped to a 4ft retort stand placed on the bench, and the outflow tubes so arranged that there was an optional communication between the lines. The pre-terminal section of each line lay within the tube that led the coolant away from the experimental chamber. This arrangement ensured that the Ringer entering the chamber was cool: the counter flow maximized the efficiency of heat exchange.

In order to assess the adequacy of the flow rate, the empty chamber was filled with a dilute solution of potassium permanganate, and the time measured for all traces of colour to be flushed out. A flow of 6ml/min achieved this within 20-30s.

Ringer Solutions

The solutions used to perfuse the preparation were modified from that used by Martin, Wickelgren & Beràнek (1970) (Table II-1). The original solution used (solution A) included a Tris maleate buffer (5mM Tris). When transmitters were to be applied in an

Table II-1

Composition of Ringer solutions (mM)

Solution	NaCl	KCl	$MgCl_2$	$CaCl_2$	$NaHCO_3$	NaH_2PO_4	Tris	Glucose	pH
A	110	2.1	1.8	2.6	-	-	5	4	7.3
B	91	2.1	15	2.6	20	0.18	-	4	7.1-7.2
C*	91	2.1	1.8	2.6	20	0.18	-	4	7.1-7.2
D	70	2.1	15	2.6	20	0.18	-	4	7.1-7.2

* Apart from the inclusion of NaH_2PO_4, this solution is identical to that used by Martin, Wickelgren & Beránek (1970).

experiment, the Ringer was modified by the inclusion of 15mM magnesium to minimize trans-synaptic effects (del Castillo & Engbaek, 1954; del Castillo & Katz, 1954).

During the course of the experiments, particularly in the summer months, it became apparent that preparations could rarely be used for more than 6-7 hours after they were decapitated. After this time it was found repeatedly that cells deteriorated before an experiment could be completed, even if the membrane potential was high when a cell was first penetrated. The whole preparation also took on an opaque appearance. In order to reduce this problem temperature control was instituted and the Ringer was modified. No attempt was made to assess the influence of the individual changes, but the overall result was a marked improvement in the useful life of the preparation.

Martin (1978, 1979a) used solution A but he, too, noted opacity of the preparation in the later stages of an experiment, and in any case did not attempt to prolong experiments beyond about seven hours (Martin, personal communication). In view of reports that several artificial buffers, including Tris, have adverse effects on other preparations (Gillespie & McKnight, 1976; Altura et al, 1980), it was decided to revert to a bicarbonate buffer as used by Martin, Wickelgren & Beràne̊k (1970) for experiments with lampreys. A small amount of sodium dihydrogen phosphate was also included, and this solution (solution B) was used for some experiments. However, the solution was hypertonic to lamprey haemolymph, especially when the magnesium concentration [Mg^{2+}] was raised (solution B). Recorded values for the osmotic pressure (freezing point depression) are

Table II-2

Ionic composition (mM)

Solution	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	HCO_3^-	$H_2PO_4^-$
Lampetra haemolymph	119.6	3.21	3.93	4.21	95.9	5.44	6.41	
Ringer B	111.18	2.1	2.6	15.0	128.3	0.0	20.0	0.18
Ringer D	90.18	2.1	2.6	15.0	107.3	0.0	20.0	0.18

Data for *Lampetra* taken from Robertson (1954).

$\triangle$0.456°C in Lampetra fluviatilis (Hardisty, 1956) and $\triangle$0.40°C in Lampetra planeri (Morris, 1958); and the ionic composition given by Robertson (1954) for Lampetra haemolymph sums to 238.7mMol/kg (Table II-2). The freezing point depression of solution B was not measured, but its ionic content exceeded 238.7mM.

It was decided to reduce the tonicity of the Ringer by adjusting the concentration of NaCl. Thereafter, solutions C or D (Table II-1) were used in all experiments. A CO_2/O_2 mixture (5:95) was bubbled through the solution continuously. These changes were beneficial: there was no evidence of a reduction in either the resting membrane potentials or the ability of the Müller cells to generate action potentials, despite the reduced sodium concentration. However some change in membrane potential may have been masked by the effects of the newly instituted temperature control.

Because it was impossible to reproduce exactly the environment in which the Müller cells exist in the living animal, the criteria used to indicate acceptable conditions were the measured behaviour of the cells and the appearance of the preparation. Solutions C and D were satisfactory in this respect. The problems of Ringer composition have been discussed in greater depth by Beament (1960) and by Lockwood (1961). Lockwood reported that inclusion of a respiratory substrate, glucose in this study, reduced the tendency of isolated tissues to lose potassium ions and to gain sodium, chloride and water; and improved the metabolic activity particularly of brain cells, these having a high turnover of ions but only a

limited store of energy. Reduction of the experimental temperature (from room temperature, 18-22°C, to 10°C) should also have helped to preserve the energy stores because it resulted in a reduced level of spontaneous synaptic activity (not illustrated).

The level of spontaneous activity was also used as the criterion when deciding by how much to raise $[Mg^{2+}]$. The solution used, containing 15mM magnesium, abolished all spontaneous potentials; but it cannot be certain that evoked activity would be similarly suppressed because this was not tested. Nor can an interaction between magnesium and the anaesthetics be ruled out. However, the results of Section IV - showing only a small stimulation of synaptic activity, and this from only two anaesthetic solutions - suggest that the contribution of any such interaction to the observed effects (Sections V & VI) would be minimal. Any changes in membrane potential measured when a drug was applied were therefore provisionally attributed to direct effects of the drug on that cell.

Fresh Ringer solutions were always prepared for an experiment, using stock solutions of the individual salts.

Recording System

Standard intracellular recording techniques were employed to measure the trans-membrane potential and the input resistance of the bulbar Müller cells.

Micropipettes were pulled from glass capillary tubing (GC 120F -15, Clark Electromedical Instruments) using a vertical electrode puller (Cat. No. 2001, Scientific & Research Instruments Ltd.).

They were back-filled with 2M potassium methyl sulphate using a syringe and 28g 2.5in needle. Filling of the tip was aided by a fine fibre present in the tube lumen.

The criteria used to assess the acceptability of an electrode were its appearance, on inspection and under a microscope, and the voltage response to injection of a constant current pulse. An electrode was rejected if the tip was broken or distorted, if air bubbles were present in the shank of the pipette, if the resistance fell outwith the range 15 to 30MΩ, if the time constant of the response exceeded 0.5ms, or if there was appreciable (>10%) rectification as evidenced by asymmetrical responses to current pulses equal in magnitude but opposite in polarity.

The properties of an electrode often altered during an experiment. An increase in electrode resistance could sometimes be reversed by briefly increasing the capacitance feedback of the circuit so as to induce oscillations. This manoeuvre, also used to effect cell penetration, is thought to cause vibration of the electrode tip, and it may have dislodged particulate material blocking the tip.

Measurement of input resistance

The input resistance was measured as the change in membrane potential produced by injection of a constant hyperpolarising current pulse through the recording electrode. A bridge circuit (Martin, 1977) was used to balance the potential change due to the electrode resistance, which was of the same order or up to 10x greater than the cell's input resistance. The bridge circuit was

switched off while the electrode resistance was being measured. Bridge circuits of different design have been described, for example by Coombs, Curtis & Eccles (1959). Both these authors and Frank & Fuortes (1955) commented on the change of electrode properties with time: the latter reported quite extensive changes in resistance when the electrode was moved short distances within the tissue of the spinal cord. Coombs et al (1959) found that the electrodes did not behave as simple resistances, especially when the injected current exceeded 30nA, or the resistance 20MΩ: this was also the case in the present investigation. However, the hyperpolarising pulses used in this study did not exceed 1.0nA. When the electrode resistance increased unduly it became impossible to "balance the bridge": this was the reason why some penetrations were abandoned while the cell showed no sign of deterioration.

Despite its limitations, the bridge method of measuring input resistance has been used in the present study because the interest was in changes in resistance rather than in its absolute value, and there was a higher probability of a successful experiment if penetration with a second electrode was not required. Even with a two electrode system, in a cell 30-50μ in diameter the tips would be sufficiently close for artefacts to be introduced into the measurements.

The high input impedance pre-amplifier incorporating the bridge circuit was built in the Department to a design previously used by Martin (1977). It was connected to the recording electrode via a Ag/AgCl wire placed in the stem of the micropipette. An Ag/AgCl

electrode (E 208, Clark Electromedical Instruments) was placed in the bath to act as an indifferent electrode. Membrane potentials were displayed on a cathode ray oscilloscope (Tektronix, 5113 Dual Beam Storage Oscilloscope) and a permanent record made using a high speed pen recorder (Lectromed, MX 212).

Criteria for satisfactory penetrations

Muller cells were penetrated under direct visual control. Most cells had resting membrane potentials in the range -60 to -65mV and input resistances greater than 3MΩ. On several occasions the penetration was not clean and was immediately followed by a rapid discharge of action potentials, considered to be an "injury discharge". Sometimes this discharge was protracted and accompanied by a continued decline of the membrane potential, which commonly had been in the range -45 to -50mV when first measured. Such cells were badly damaged and were not used. In other cases the discharge of action potentials ceased within 1-2min, and there was then a recovery of the resting potential and a gradual increase in input resistance. When their membrane properties had stabilised it was considered admissable to use these cells for experiments, as it was likely that the observed phenomena could be explained by a gradually improving seal of the surface membrane around the micropipette. Further evidence of the satisfactory condition of cells was the observation at high gain of spontaneous synaptic potentials (see Section IV).

Application of Neurotransmitters

Apart from the preliminary tests, in which amino acids were applied to the preparation dissolved in the perfusate, the technique

of microiontophoresis (del Castillo & Katz, 1957; Curtis, 1964) was used. This decision meant that the better control of transmitter concentrations afforded by the former method was sacrificed in the interests of allowing an experiment to be completed within a reasonably short time span. However, even with bath application of transmitter, the concentration achieved at the receptors cannot be known accurately because of the uncertain influence of uptake mechanisms. Prolonged bath application would not allow measurement of the peak responses if there were any desensitisation process.

3-barrelled glass micropipettes were routinely used for iontophoresis. These were pulled using another vertical electrode puller (S.R.I.) from fused capillary tubing (Clark Electromedical Instruments, 3GC 120F-15). Use of this technique resulted in an assembly in which the barrels were parallel but the tips, although separate, were closely adjacent. The pipettes were back-filled in the same manner as were the recording pipettes. It was feared that such close apposition of the tips would lead to the spread of solution between barrels, but when only one barrel was filled liquid was rarely drawn into the other two tips. Furthermore, no difference in the response to a transmitter was detected which could be related to whether the three barrels were filled with the same or different solutions, or two barrels were left empty. Electrodes in which contamination was known to have occurred were rejected, as were any that were improperly filled or whose tips were broken. Iontophoretic currents employed in the experiments rarely exceeded 200nA, but each barrel used was capable of passing 800nA for 5s. If no current was passed when the electrode was first tested, the tips

were either broken back under a dissecting microscope using a soft tissue, or, if only one channel was affected, that channel was not used. An electrode was discarded if repeatable pulses could not be obtained. If one channel ceased to pass the current set during an experiment, the data for the transmitter affected were discarded.

Before being used, an iontophoretic electrode was held vertical with its tips in Ringer for at least 30min to allow a degree of equilibration at the electrolyte-Ringer interface. This was done so that regular pulses applied to the barrels would result in the ejection of standard pulses of transmitter. Electrodes that were still performing well at the end of an experiment were saved for re-use. In the interim they were stored held vertical with their tips in distilled water, and covered to reduce evaporative losses. This procedure did not result in detectable mixing of the solutions, and on occasion an electrode was used on 5 successive days.

The solutions used were:	GABA	0.5M	pH 2.5
	Glycine	0.5M	pH 5.0
	L-glutamate	2M	pH 8.5
	NaCl	0.5M	pH 7.0

GABA and glycine were ejected as cations and glutamate as an anion.

Retaining currents were not routinely employed. Any leakage of drug was insufficient to cause detectable changes in the membrane potential or resistance of the cells, and the detection of such changes would in any case have been the criterion by which a retaining current was judged adequate. In view of Bradshaw &

Szabadi's conclusion (1974) that the diffusional release of a drug from the pipette tip was best countered by the use of dilute drug solutions, low concentrations (0.5M) were used when possible. However, glutamate responses of reasonable amplitude were recorded only when the electrode was filled with a 2M solution.

It was necessary to estimate the direct effect of the iontophoretic current on the Müller cells. Pulses of reverse polarity were therefore applied to the drug-containing barrels - invariably with no detectable result other than that artefacts were recorded at the onset and offset of each pulse. Since this was done with all three transmitters, the lack of response was demonstrated both to cationic and anionic currents. In addition, NaCl was incorporated as a control in some experiments. In all experiments an Ag/AgCl indifferent electrode was placed in the bath for the return of iontophoretic current.

The ejection cycle was controlled by a micro-iontophoresis programmer (Model 160, W-P Instruments Inc.) driven by a Digitimer (D100). The three transmitters were applied in rotation by currents that either were of the same strength or differed by a factor of 10. GABA and glycine were usually ejected by currents in the range 50 to 90nA whereas higher currents (100-400nA) were frequently needed to elicit glutamate responses: a x10 switch present on the programmer accommodated this difference. Pulses lasting between 0.3 and 7.0s were applied at intervals of 1 or 3min. The pulse length was determined for each transmitter individually and then kept constant for the duration of an experiment.

In experiments testing the influence of transmitter concentration on the anaesthetic effect only one transmitter was used. To vary the transmitter dose a cycle of pulses of differing durations but the same amplitude was employed. The quantity of drug ejected by an iontophoretic pulse is affected by the immediately preceding pulse, but the use of a fixed pulse sequence ensured that this factor was unchanged during an experiment. Differences between the amounts of the transmitter delivered by the test pulses were therefore constant although unknown, so measurements of the responses are comparable but only within an experiment.

Both drug emission and retention are time-dependent processes (Bradshaw & Szabadi, 1974), so there cannot be a retaining current that is always just adequate. Furthermore, the relation between the pulse length and the quantity of drug emitted must be non-linear, particularly with short pulses. Therefore any dose-response curve constructed in Sections V and VI must have a non-linear scale for the abscissa (dose). However, use of the alternative method of varying the dose, that of varying the pulse amplitude, would have resulted in less reproducible doses and no gain in linearity - unless pulses long enough to establish a plateau rate of release were employed. The latter would however raise again the possibility of receptor desensitisation.

Iontophoresis techniques are limited in that the concentration of drug achieved at the receptor can only be estimated. Had perfusion not been continuous, it would have been possible to make some estimate by comparing the responses to identical pulses before

and after a known movement of the electrode. However, the micro-manipulators were calibrated only for vertical movements and these were not always in a plane normal to the cell surface. Furthermore the complex cell architecture invalidates the implicit assumption of a "point" receptor. Additional complications were introduced by the existence of eddy currents caused by the obstruction which the preparation presented to the flow of Ringer.

The effects of anaesthetics on transmitter responses

Anaesthetics were applied to the preparation dissolved in the perfusing Ringer solution. No application was continued after 25min; this limit was imposed to allow the responses to recover before cell deterioration supervened. The sequence of iontophoretic pulses continued during the perfusion with anaesthetic. Changes in input resistance associated with the transmitter actions were measured by injecting constant hyperpolarizing current pulses (strength 0.5-1.0nA; frequency 1Hz) whose duration was long (30ms) in relation to the time constant of the cell (<10ms).

Penetrations were held for periods up to three hours, so application of several concentrations of an anaesthetic to a single cell was sometimes possible. When this was done, the solutions were applied in order of increasing concentration, with or without an intervening wash. Because of the possibility that anaesthetic drugs may interact (Richards & White, 1981), only one was tested on any individual preparation. Most experiments were terminated within ten hours of dissection.

Drugs

The drugs used were GABA (Sigma), L-glutamic acid (BDH), glycine (BDH), substance P (Sigma), tricaine methane sulphonate (MS-222, Sandoz), pentobarbitone (May & Baker), ketamine (Vetalar, Parke, Davis & Co. Ltd.), alphaxalone/alphadolone (Saffan, Glaxovet) and metomidate (Hypnodil, Janssen). The vehicle present in Saffan was kindly donated by Glaxovet. AnalaR grade reagents were used in the preparation of Ringer solutions.

The veterinary medicinal product Vetalar contains:

Ketamine hydrochloride	100mg/ml
Benzethonium chloride	1:10,000.

The ketamine base (di-2-(o-chlorophenyl)-2-(methylamino) cyclohexanone) is 86.7% of the salt. Benzethonium chloride is included as a preservative and the product is supplied as an acid solution (pH 3.5-5.5).

Analysis of results

The responses to GABA and glycine were essentially similar. Numerical values were obtained for the peak resistance changes as indicated in Figure II-6A. A videoplan (Reichert-Jung) was used to measure the time integral of the membrane potential response (area "X"). Figure II-6B shows how the ratio of this area to the peak hyperpolarisation distinguishes between an increase in area due solely to an altered peak amplitude and one effected by prolongation of the response. The ratio was calculated in experiments in which pentobarbitone or ketamine were tested, because these drugs altered the GABA response profile.

Figure II-6

A. Tracing of a typical response to an iontophoretic pulse of GABA. Vertical deflections (downward) from the resting membrane potential are in response to repeated injections of hyperpolarising current pulses (1nA, 30ms). GABA was applied during the period indicated by the horizontal bar.

Features measured: 1) peak change in membrane potential $(V_2 - V_1)$,

2) membrane potential change in response to hyperpolarising current pulses before $(V_3 - V_1)$ and during $(V_4 - V_2)$ action of GABA. The height of each vertical deflection is directly proportional to the input resistance. Because constant current pulses were used, the fractional change in input resistance at peak GABA effect is

$$\frac{(V_3 - V_1) - (V_4 - V_2)}{(V_3 - V_1)}$$

3) area (X) enclosed by the envelope of the membrane potential response.

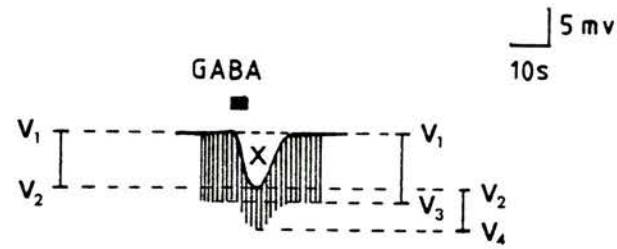
B. Diagram to illustrate use of measurement of ratio area : peak. Control (- - -) and test (——) responses are superimposed.

(i) Simple potentiation of membrane potential response.

(ii) Alteration of response profile showing both delayed peak response and slowed recovery.

In both (i) and (ii) the area of the response is increased from 9mV.s (control) to 19mV.s (test); but whereas in (i) the ratio remains 3.5s, that in (ii) is increased from 3.5s (control) to 7.2s (test).

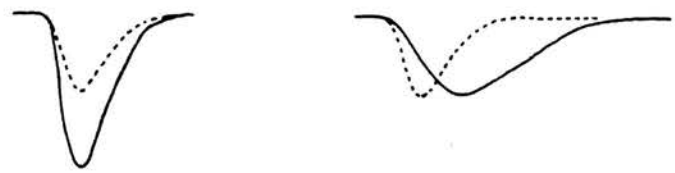
A



B

i.

ii.



In contrast to the hyperpolarising GABA and glycine responses, those to glutamate were invariably depolarising. Fewer measurements were made on the glutamate responses. The change in input resistance was often barely detectable and could not be measured with any accuracy. Furthermore there was no visible change in the response profile following perfusion with anaesthetic so the area:peak ratios were not calculated.

There was considerable variation between cells in the absolute magnitudes of the responses, particularly in the membrane potential measurements. Therefore, before different experiments were compared, their results were normalised by expressing the change in a GABA or glycine response measured during exposure to the anaesthetic as a percentage of the mean control value in that cell. Student's t-test was applied to these standardised data to assess the statistical significance of the anaesthetic effects. The null hypothesis used was that the proportional difference between the mean response amplitudes in the control period and in the presence of anaesthetic did not differ from zero. The numerical measurements and significance values are shown in Appendices I & II. The results are only semi-quantitative and the pictorial display of the collected results given in Sections V and VI is probably more helpful.

Two-way analysis of variance (AOV) used in conjunction with the F test is a more powerful method of analysing data of this sort and has been used in experiments on preparations, such as the neuro-muscular junction, where there is greater uniformity of the control values. However, it is not applicable in the present case because

normalisation of the results removes all variance from the population of control readings, thereby invalidating the test. Application of AOV to the raw (unstandardised) data caused some dramatic results to appear insignificant: this occurred when the differences between the control responses of different cells were greater than the absolute values of the anaesthetic effects (see Appendix III). Those effects shown as significant in Appendix III were genuine effects of the anaesthetic: when the "main" (anaesthetic) effect was tested all sources of variation were taken into account in calculating the variance ratio. The further objection, that the change in response had not always reached equilibrium, applies also to the t-test; but in the latter this factor should tend to reduce the significance of anaesthetic effects.

SECTION III

Anaesthesia in lamprey ammocoetes, as produced by injectable compounds

Introduction

> ...a small flounder was introduced into a vessel filled with solution of nitrous oxide in water over mercury. He remained at rest for ten minutes and then began to move about the jar in different directions. In half an hour he was apparently dying, lying on his side in the water. He was now taken out and introduced into a vessel filled with water saturated with common air, he very soon recovered...
>
> Davy (1800)

The term "anaesthesia" has received many definitions since it was first coined by Dioscorides in the 1st Century A.D., but in essence it is the reversible loss of conscious awareness of, and of motor responses to, external stimuli including strong and noxious stimuli. The extent to which a lamprey is consciously aware of its surroundings is debatable; so therefore also must be the contention that a lamprey can be anaesthetised. However lampreys, like fishes, respond to light and vibratory stimuli, and several studies have been made of anaesthesia in fishes (e.g. McFarland, 1959; Oswald, 1978; Stuart, 1981).

Each of the drugs used in this study has been reported to anaesthetise fishes (see Stuart, 1981). The experiments described in this section were carried out to test whether this was true also in lamprey ammocoetes. Previous neurophysiological and neuropharmacological studies have suggested that the lamprey brainstem behaves in a similar manner and is organised along similar lines to those of higher vertebrates (Section I). If extrapolations from the actions of anaesthetics on transmitter responses in the lamprey are to have any relevance to events occurring during anaesthesia in higher animals, it is essential that it be known that the drugs anaesthetise lampreys, and at what concentrations this occurs.

Otherwise the argument that neurones of the lamprey brainstem may differ pharmacologically from those of other species is hard to refute.

Methods

Ammocoetes within the size range 40 to 120mm were used in the experiments. A treatment group (three ammocoetes) was rarely exposed to more than one drug concentration during an experiment, and never to more than one drug. In the preliminary tests the anaesthetics were dissolved in Ringer solution C (1.8mM Mg^{2+}): subsequently fresh stream water was used. Ammocoetes were exposed to the same concentrations of the drugs as those used in the experiments of Sections IV, V and VI.

Before exposure to an anaesthetic, all groups were left at room temperature for an hour to permit the water temperature to stabilise - both the stream water and the lampreys were stored at 4-6°C. At the beginning of the test period each group was transferred to a fresh vessel containing a 250ml volume either of an anaesthetic solution or of stream water. A watch was kept on the unstimulated activity of each ammocoete. Anaesthesia was classified according to the scheme proposed by McFarland (1959) and since widely accepted (see Table III-1). Comparable stimuli were used to test the reactivity of the ammocoetes: a flashing light; tapping the rim of the vessel in order to set up vibrations in the solution, and sometimes direct agitation of the solution to create a similar but stronger stimulus; and mechanically stimulating the ammocoetes by pinching their tails with forceps.

Table III-1 Stages of anaesthesia in fish

(Adapted from McFarland (1959): based on 3 species of marine teleost fish.)

Anaesthetic stage	General activity	Balance	Responses to stimuli visual	Responses to stimuli vibrational	Opercular movements
0	variable (characteristic)	normal	+ +	+ +	normal
I-1	reduced (more hovering)	normal	+	+ +	normal
I-2	mainly hovering	normal	-	+/- early: rapid darting late: barely perceptible	normal
II-1	random uncoordinated movements	partial loss	-	+/- occasional darting	increased
II-2	no swimming react only if pinched	complete loss	-	+/-	rapid decline amplitude unchanged
III	no movement	complete loss	-	-	slow, exaggerated
IV	no movement, no responses to strong stimuli	complete loss	-	-	cease, opercles spread cardiac arrest later

At the end of the test period the lampreys were transferred to fresh stream water, and observations continued. In most cases the exposure was limited to 30min; but the effect of the highest concentration of alphaxalone (2.7×10^{-4}M) was so profound that the ammocoetes were removed after 5min (and after 10min from the next lower concentration) in order to prevent unnecessary deaths. Exposures to (10^{-3}M) pentobarbitone and (3.7×10^{-5}M) ketamine were prolonged to 60min in case the anaesthetic effects of these solutions were slow in developing.

Each drug was tested on a minimum of 6 ammocoetes. This number was increased when the response was in doubt, as was the case with pentobarbitone and the lower concentrations of ketamine. The variable replicate numbers which resulted were accepted because no statistical analysis of the results was envisaged. Most experiments were conducted at room temperature (water temperature 15-18°C), but alphaxalone was also tested at 4°C, with similar results. An accurate record of temperature was not kept. The preliminary experiments, in which anaesthetics were dissolved in Ringer, were performed during June and July when the ambient temperature was high; the solution temperature then may have exceeded 18°C. It is recognised that both the rate of induction and the depth of anaesthesia in fishes are affected by temperature, the induction time commonly being shorter at higher temperatures (discussed by McFarland, 1959; Houston & Woods, 1976). Notwithstanding this, a strict acclimatisation routine was not employed because of the essentially qualitative nature of the study. In any case, the issue of possible temperature effects is peripheral to the main object of

these experiments.

Pentobarbitone was tested also by direct systemic injection to three ammocoetes. The ammocoetes were blotted and then weighed on an Oertling balance. The dose for each individual was calculated on a body weight basis and administered through a 28g needle as an intra-peritoneal injection. A proprietary solution diluted 1:1000 with 0.9% saline was used (Sagatal, May & Baker; containing pentobarbitone 60mg/ml). A fourth lamprey was injected with a similar volume of 0.9% saline as a control. The three ammocoetes received different doses of pentobarbitone:

1) 15mg/kg;

2) 35mg/kg;

3) 15mg/kg followed after 15min by 30mg/kg;

and the volume of injection was of the order of 0.1ml. This was a relatively large volume for an ammocoete weighing only about 1.2g.

Ammocoetes tolerated the procedures of weighing and injection without undue resistance so prior sedation by bath application of an anaesthetic such as tricaine methane sulphonate was considered unnecessary. Use of a sedative might have masked some of the effects of the injected pentobarbitone, although Oswald (1978) claimed otherwise in his studies on trout. Restraint for injection was achieved by covering the anterior portion of the ammocoete with a swab wet with stream water, the animal lying on a smooth surface. Following the injections, the ammocoetes were transferred to fresh water and maintained in isolation for observation.

Results

The results of the experiments in which anaesthetics were bath-applied are presented in tabular form in Table III-2. McFarland's scheme, used to classify the stage of anaesthesia reached at fixed time intervals after exposure to a drug, is shown in Table III-1. The behaviour of the ammocoetes fitted his description of anaesthesia in fishes in general terms. The effects of the anaesthetics are described separately below. No deaths ocurred during this series of experiments.

Unstimulated ammocoetes exhibited a variety of behaviour patterns that were characteristic yet are not readily described. Once accustomed to the vessel the ammocoetes did not break the surface of the water. Periods of quiet swimming alternated with others when the ammocoetes lay on the bottom of the vessel dorsal surface uppermost or, around the perimeter, with the dorsum against the side of the vessel. Rapid movements were rare in unstimulated ammocoetes. In control animals, both light and vibratory stimuli caused an increase in the general level of activity. This observation contrasts with McFarland's finding that visual stimuli caused fish to sink to the bottom of the tank in a "fright" reaction. Pinching the tail elicited a violent lashing escape movement which usually freed the tail from the forceps; when escape was impossible, the ammocoetes coiled back against the forceps. A period of rapid swimming ensued which lasted for up to one minute. It was notable that although forward progress was fast, the waves of contraction passing down the body were relatively slow.

Breathing movements could be counted reliably only when the

Table III-2

The effects of injectable anaesthetic drugs when bath-applied to intact lampreys

Drug	Concentration (M)	Stage of anaesthesia reached after various time intervals			
		2min	5min	10min	30min
Pentobarbitone	10^{-3}	-	-	I	I
Ketamine	3.7×10^{-5}	-	I	II-1	II-1
	3.7×10^{-4}	I	II-1	II-2	II-2
	3.7×10^{-3}	II-1	II-2	II-2	III/IV
Alphaxalone	2.7×10^{-6}	-	-	I	II-2
	2.7×10^{-5}	II-1	II-2	II-2	
	2.7×10^{-4}	II-2	III	III/IV	
Metomidate	1.8×10^{-5}	-	-	I-2	I-2
	1.8×10^{-4}	-	II-1	II-2	II-2
	1.8×10^{-3}	II-2	II-2	II-2	

Drugs dissolved in river water (unbuffered), times measured from moment of transfer of ammocoetes to anaesthetic solution. Classification of anaesthesia after McFarland (1959), tabulated stages are the averages for the group (3-8 ammocoetes).

lampreys were still, but they appeared to be more frequent during swimming. This indicated that the level of activity should be taken into account before attributing a change in breathing rate to a direct effect of the drug under test.

Pentobarbitone

Pentobarbitone was unique in not inducing anaesthesia deeper than Stage I when bath applied in any concentration. Even 10^{-3} M pentobarbitone produced only mild sedation in 8 ammocoetes.

Intraperitoneal injection proved more effective: these results are presented in Table III-3. Because the ammocoetes were separated, measurement of the breathing movements was easier than in the other experiments, and more readings were obtained. These observations were not in accord with those of McFarland, pentobarbitone acting as a greater respiratory depressant in lampreys than would be predicted from the level of anaesthesia induced. For example, comparison of Table III-1 with Table III-3 reveals that Stage I anaesthesia was accompanied by a marked fall in breathing rate in all three lampreys, but not in fish. Deep sedation was produced by the higher dose levels - 30mg/kg (following 15mg/kg) and 35mg/kg - but surgical anaesthesia was probably never achieved.

No evidence of excitement was seen during either induction or recovery; but there was a period (e.g. 20-40 minutes after receipt of 15mg/kg pentobarbitone) when stimulation evoked exaggerated responses, the lampreys seemed restless and non-propulsive movements occurred. These movements characteristically were composed of contraction waves which were more frequent and of shorter wavelength

Table III-3

The anaesthetic effect of pentobarbitone administered by systemic injection

Pentobarbitone:	15mg/kg		30mg/kg*		35mg/kg	
Exposure time (min)	Stage	Breathing rate	Stage	Breathing rate	Stage	Breathing rate
1				48		86
3		88				
5			I	48		
10	I	58			I	42
15			II-1			
20	II-1			54	II-1	
30	II-1		II-1/2			
40	II-1				II-1/2	
45				56		
50	II-1	36				
60	I	34		55	II-1	67
70	I	48				
80	0	42	0			
100	0	46			0	

* following 15min after a dose (15mg/kg) which had no apparent effect

Classification of anaesthesia after McFarland (1959); tabulated rates are breathing movements per minute.

than those occurring in the resting state or in the control animal. The contraction waves passed both rostrad and caudad. Sedated ammocoetes spent more time than did the control animal lying on the bottom of the vessel. Nevertheless, even after their response to a tail pinch was markedly reduced by 30mg/kg pentobarbitone, two lampreys swam for short spells in a slow and uncoordinated manner. Recovery was evidenced by restoral of the escape reaction to its previous brisk nature.

Ketamine

The lowest concentration (3.7×10^{-5} M) was tested on 15 lampreys. There was no excitement evident and only mild sedation was induced. By about twenty minutes there was some loss of equilibrium and incoordination apparent in all ammocoetes, but movement was still stimulated by persistent vibratory or light stimuli.

When first tested, dissolved in Ringer, this concentration of ketamine had induced Stage II-2 anaesthesia within ten minutes in all three ammocoetes, the lampreys making only feeble attempts at escape when the tail was pinched quite hard. The failures of attempts to repeat this observation may have related to temperature differences - the preliminary experiments were carried out when the ambient temperature was high (in July) whereas the majority were performed during the winter months. Two batches of ketamine were used on the second occasion so the difference cannot be attributed to loss of potency of the solution. The tabulated results (Table III-2) are those obtained using the new batch. A further

preliminary observation had been that immediately on exposure to ketamine all ammocoetes showed signs of irritation: rapid twitching and lashing movements, rubbing against the side of the vessel and attempts to leap out. This may have been the response to irritation by acid (ketamine is supplied as the hydrochloride, in acid solution). However, the point was not proved: when the drug was tested more fully neither were the solutions very acid (see Table III-4) nor did they have an irritant effect. It is interesting that Ohr (1976) found neutralised tricaine methane sulphonate to be more potent as an anaesthetic of goldfish and frogs, and to cause less stress, than when it was tested in acid solution.

After five minutes exposure to 3.7×10^{-4}M ketamine the responses to both light and vibratory stimuli were much reduced. However, the brisk escape reaction was retained, and 2 out of 8 ammocoetes still swam spontaneously.

In contrast, 3.7×10^{-3}M ketamine abolished spontaneous movements within five minutes, and by fifteen minutes all ammocoetes responded to a squeeze of the tail with only a brief and feeble twitch. At thirty minutes this response also was lost. Recovery from anaesthesia was uneventful and within forty minutes the ammocoetes could be roused, some of them also swimming spontaneously.

Alphaxalone

The immobilising effect of the most concentrated solution used (2.7×10^{-4}M) was instant and dramatic. All ammocoetes immediately sank to the bottom, and although initially they twitched in

Table III-4

The reaction of ketamine solutions and their effects on breathing rates

Ketamine	3.7×10^{-5} M	3.7×10^{-4} M	3.7×10^{-3} M
pH	7.2	6.9	5.2
Breathing	96, 84	62	
	(96, 120, 120)	(112)	

Breathing rate (per minute) measured during action of anaesthetics; individual measurements quoted, with recovery values in parentheses.

response to a tail pinch, there was no real attempt at escape. Two minutes later this response had failed and the opercular rate was reduced to between 14 and 28 per minute.

The onset of action of the more dilute solutions was gradual. Within five minutes of first immersion in 2.7×10^{-5} M alphaxalone there was no response to vibratory or light stimuli. A brisk reaction occurred when the tail was pinched but this was not followed, as in the control group, by a period of swimming. The twitch response was more sluggish after a further five minutes, and the opercular rates recorded in two individuals were now 12 and 18 per minute. Exposures to both the higher concentrations of alphaxalone were restricted to ten minutes.

The lowest concentration, 2.7×10^{-6} M, was tested in the expectation that anaesthesia would not be induced, since the estimated concentration of alphaxalone in the brain during surgical anaesthesia is 8×10^{-5} M (Table III-5). Nevertheless, it was evident after ten minutes that the general level of activity was reduced as were the responses to stimulation, and by twenty minutes the anaesthesia was classified as Stage II-2: pinching the tail elicited only a twitch.

No hyperactivity was observed during induction with any concentration of alphaxalone, but during recovery there was a period when uncoordinated movements occurred: both rolling and other non-propulsive movements. Exaggerated reactions to stimulation, such as have frequently been reported to follow alphaxalone anaesthesia in mammals (see Section I) were not detected; nor were convulsions

Table III-5

Estimated brain concentrations of anaesthetics during clinical use in fish

Anaesthetic	Dose	Brain Conc. (M) (Estimate)	Effect on Fish	Ref.
Pentobarbitone	30mg/kg i.p.	1.1×10^{-4}	sedation	1
	48mg/kg i.p.	1.8×10^{-4}	apnoea	1
Ketamine	130mg/kg i.m.	9.5×10^{-5} *	surgical anaesthesia	1
Alphaxalone	18mg/kg i.p.	8.1×10^{-5}	surgical anaesthesia	1
Metomidate	7.5mg/l (bath)	2.8×10^{-5} *	surgical anaesthesia	2

* upper limit 1 = Oswald (1978) 2 = Stuart (1981)

Assumptions (Minchin, 1981): rapidly acting compounds (alphaxalone, metomidate) distributed in E.C.F. (= 30% body weight); slowly acting compounds (pentobarbitone, ketamine) distributed in total body water (= 66% body weight); protein binding 40% for alphaxalone and pentobarbitone.

elicited. Muscle tremors were not observed during the period of anaesthesia. Recovery was slow, in contrast to the rapid induction, and after ammocoetes were removed from the more concentrated solutions it was an hour before there was even a slight response to a tail squeeze.

Metomidate

All concentrations of metomidate tested had some sedative effect on the ammocoetes, although in the case of 1.8×10^{-5}M this was limited to a slowing of the responses to stimulation: intermittent swimming still occurred. The two higher concentrations both had an apparently irritant effect: immediately on being placed in the solution the ammocoetes exhibited rapid thrashing movements, rubbing against the side of the vessel and making as if to jump out. This phenomenon was not investigated further, and the experiments were not repeated with neutralised solutions because the objective, to establish that metomidate could anaesthetise lampreys, had been achieved. The behaviour of the ammocoetes was, however, very similar to that just described for ketamine, and to that regularly observed if an unbuffered (acid) solution of tricaine methane sulphonate was used to anaesthetise lampreys prior to their decapitation. Ammocoetes picked out of the 1.8×10^{-3}M solution were noticeably more rigid than the limp ammocoetes in the 1.8×10^{-4}M solution.

Recovery from metomidate anaesthesia was notable for its rapidity. The three ammocoetes removed from a 1.8×10^{-3}M solution, where they were anaesthetised so deeply that a hard squeeze elicited only a slight movement, were swimming within thirty minutes of their

return to fresh water. Recovery from the effects of a 1.8×10^{-4} M solution took only between ten and twenty minutes.

Discussion

Anaesthesia in lampreys: their behaviour compared with that of higher vertebrates

McFarland (1959) stressed the remarkable parallelism that exists between anaesthesia in fishes and that in man, and concluded that in both cases anaesthesia was produced by the sequential depression of functionally equivalent nerve centres in the brain. Anaesthesic stages were defined according to the brain functions that were suppressed.

In this study, a comparable progression of stages has been found in lamprey ammocoetes, such that McFarland's classification scheme can be applied. The chief deviations of lamprey behaviour from the scheme were in the nature of the "fright" response of fishes to visual stimulation, and in the effects of anaesthetics on breathing movements. These are points where McFarland, too, noted some inter-species variations. The lampreys exhibited nothing comparable to the fright response: stimulation, whether by light or vibration, served merely to increase the level of activity. Such responses are appropriate in the field, where agitation of the water causes lampreys either to retreat into their burrows or, if they are freely swimming, to increase their activity so that they can maintain their position or reach quieter waters. Similarly, an increase in activity in response to light stimuli can be seen to favour lampreys' movement into shady areas, and so constitute a

further adaptation to the burrowing lifestyle (Hardisty, 1979). A fright response may require a higher level of integrative activity than is present in the lamprey brain.

It is clear from Tables III-2 & III-3 that all four drugs tested have anaesthetic activity in lampreys. Reference to Table III-5 shows that, with the exception of pentobarbitone, concentrations likely to obtain in the brains of fishes during clinical anaesthesia were capable of inducing anaesthesia. The equivalent figures for mammals (not shown) are of the same order.

Effective brain concentrations of the anaesthetics

The precise relation of anaesthetic concentrations in the CNS to those in the water is uncertain. A dynamic equilibrium must ultimately be reached between the various phases: external bulk solution, solution at the absorptive surface, the blood, extracellular fluid and the various tissues and membranes of the body. The length of time required for equilibrium to be achieved is illustrated by the experiments using alphaxalone: a low concentration (10^{-6} M) initially had no apparent effect, but by twenty minutes the ammocoetes were in Stage II-2 anaesthesia. This result was surprising since the calculated CSF concentration of alphaxalone during surgical anaesthesia in mammals is of the order of 8×10^{-5} M (Table III-5). However, in clinical practice alphaxalone is usually given by i.v. bolus injection. Peak levels are not sustained sufficiently for there to be equilibration within the brain because elimination mechanisms are operative from very shortly after the moment of injection. Much lower concentrations are used in continuous infusion techniques. In this connexion it is

interesting that Richards & Hesketh (1975) were rarely able to expose olfactory cortex slices to 5 x 10^{-5} M alphaxalone for more than twelve minutes without causing irreversible loss of the responses to the excitatory transmitters glutamate and acetyl choline.

Factors affecting the anaesthetic potencies of drugs in poikilotherms

The experiments in this section exemplify several points of practical import to the anaesthetist working with aquatic poikilotherms. Factors such as the oxygen content, temperature and hardness of the water are important determinants of the narcosis produced by a given dose. This observation, in addition to that of species differences in sensitivity to noxious stimuli, led Ollenschloeger (1975) to point out that general guide-lines, only, can be given for dose-rates appropriate in fish. Such factors operate by varying the availability of a drug to the absorptive surface or, by metabolic means, by affecting the rate of elimination of the drug. The routes by which drugs can be absorbed include the skin and the gill system. Little is known about the former, but Hunn & Allen (1974) have reviewed the literature concerning absorption by the gills of fishes. Among the drugs they considered was the lampricide "TFM" and, although they did not discuss its basis, the existence of a selective toxic effect against lampreys raises the possibility that the barrier to drug absorption is less in lampreys than in fishes.

Factors affecting the rates of absorption of drugs do so by

altering the trans-membrane concentration gradient or the permeability of the absorptive membrane. They include the temperature, pH and ionic composition of the external solution, as well as the presence of any potential drug chelators or compound that may alter the fixed electrical charges on the membrane surface. Thus Houston & Woods (1976), working with rainbow trout (Salmo gairdneri), found that the blood level of tricaine methane sulphonate at the onset of anaesthesia was independent both of temperature and of the body weight, despite the fact that the concentration measured in the brain varied with temperature (that at 5°C was lower than that at 15°C). Factors pertaining to the test animal are also important; in particular, the permeability of the gill membranes and the blood flow to the gills. For example, tricaine methane sulphonate reduces gill blood flow by up to 24% (Houston & Woods, 1976), a self-limiting action, and other anaesthetic drugs may have comparable effects, as they do on the mammalian circulation (e.g. Vickers, Wood-Smith & Stewart, 1981). The gill system of the lamprey presents but little barrier to diffusion - two layers of cells, a basement membrane, and the flanges of the pillar cells which enclose the blood spaces. The total thickness of this barrier has been estimated at 4.57 μm in the ammocoete (Lewis, 1976).

Temperature

The effects of temperature on the induction of anaesthesia in poikilotherms are complex, and involve several stages in the process. Temperature affects the activities of a wide range of enzyme systems: a typical Q_{10} for an enzymic reaction is 2. Of

direct importance to anaesthesia are those enzymes acting as ion pumps to maintain the trans-membrane ion gradients and thus the membrane potential. In addition, recent measurements of the properties of single ion channels have shown that the open channel lifetime is increased at low temperatures, at least in the case of chloride channels operated by GABA and glycine. The depolarising effect of a fall in temperature is illustrated in Figure II-5, and must have repercussions on the excitability of excitable membranes. Other transport systems, and therefore membrane permeabilities, may also be affected, as may those enzyme systems that determine the rate of elimination of a drug.

However, poikilotherms have been shown partially to compensate for variations in temperature by making metabolic changes (Bullock, 1955). This observation was put forward by McFarland (1959) to explain the discrepancies he observed between anaesthetic potencies measured at different temperatures. The potency difference at two temperatures was less if the fish were acclimatised to each temperature tested, rather than being subjected to sudden changes in temperature during the course of an experiment. Induction times were generally less at high temperature. The effect of temperature depended on the drug concerned and reflected the means by which it was eliminated. Variations in temperature are likely also to affect the physical properties of an anaesthetic solution, such as the mobility of the drug, and thence also the availability of anaesthetic to the absorptive surface.

In the present study lampreys were routinely stored at 4-6°C, and before being used for an experiment, were left for an hour at

room temperature so that the water temperature stabilised. Since the results were essentially qualitative no detailed temperature record was kept; but with the exception of ketamine, the recorded anaesthetic levels were consistent to within a substage, irrespective of the ambient temperature. As a further check, alphaxalone was also tested at 4°C and was found to induce a similar quality of anaesthesia then to that previously induced at room temperature.

Pentobarbitone-calcium interaction

The anomalous behaviour of pentobarbitone was consistent with previous observations on fishes, notably those of McFarland (1959) and Oswald (1978), and with their conclusion that bath application of the barbiturates is not a clinically useful procedure. McFarland attributed the failure of barbiturates to anaesthetise marine teleosts to an antagonistic action of the calcium in sea water; observing that the anaesthetic effect of sodium amytal on Fundulus was greater in fishes adapted to fresh water. Addition of a calcium chelating agent, citrate, increased both the rate of induction and the depth of anaesthesia in fresh water (Ca^{2+} 0.28-0.30mM), although not in sea water (Ca^{2+} 8.8-8.9mM) or fortified distilled water (Ca^{2+} 0.04mM). The mechanism of this calcium antagonism is unclear but could involve complex formation with the barbiturate base, or a direct effect of calcium on barbiturate uptake by the gills.

The hardness of the water used to dissolve a barbiturate is thus of prime importance. Water used in the present investigation was soft, obtained from local streams which had granite as the bed-

rock. A sample of the fresh water had a total calcium content of 0.106mM, whereas one taken from a tank in which lampreys had been stored for some weeks had 3.8mM total calcium. These concentrations, like that in the Ringer solution used (2.6mM) were in the range where addition of a chelator might have an observable effect. This theory, however, was not tested.

Oswald (1978), administering pentobarbitone to trout by intraperitoneal injection, found that whereas 30mg/kg produced sedation, 48mg/kg caused apnoea and 72mg/kg induced protracted anaesthesia. Only 4 fish received pentobarbitone in that study, and only 3 lampreys received injections in the present experiments, but the results were consistent. The apnoea mentioned by Oswald has a counterpart in this study in the recorded slowing of the opercular movements (Table III-3). Protracted recovery was not noted with lamprey ammocoetes, nor was persistent ataxia; but the dose rates were lower, and Oswald did not specify the duration of the sedative effects.

SECTION IV

The effects of anaesthetics on spontaneous synaptic activity recorded in bulbar Müller cells

Introduction

The experiments reported in Section III demonstrated that the drugs used in this investigation are able to induce a state of reversible loss of responsiveness (anaesthesia) in lamprey ammocoetes. Because the experiments that form the bulk of this dissertation were concerned not with the whole animal but with the responses of the bulbar Müller cells to exogenous putative transmitters, it was of interest to test also whether the anaesthetic drugs influenced the spontaneous synaptic activity recorded from these cells.

Abnormal patterns of activity were to be expected because severance of cranial nerves and of the spinal cord produced partial deafferentation of the Müller cells. Spinal section must also have transected the giant axons, and there may have been other dissection damage. However, other afferent connexions to the cells were essentially undisturbed so it was likely that, while a preparation remained viable, some normal synaptic activity would continue.

Small spontaneous potentials, both hyperpolarising and depolarising, have been recorded from Müller cells (Rovainen, 1967; Martin, 1977). Because all were eliminated when the magnesium content of the bathing solution was raised to 10-15mM it was inferred that their origin was trans-synaptic: a decrease in the calcium-magnesium ratio in the external solution is known to inhibit the release of transmitter from pre-synaptic nerve terminals (del Castillo & Engbaek, 1954; del Castillo & Katz, 1954). I have not considered it appropriate to make the detailed analysis of these potentials a major feature of this investigation. In some

preparations it is now possible to analyse synaptic events at the level of single ion channels, using biophysical techniques such as voltage and current fluctuation analysis and patch clamping (for review see Mathers & Barker, 1982). Gold & Martin (1982b) have recently applied noise analysis to the lamprey preparation and have interpreted spontaneous inhibitory post-synaptic currents (IPSCs) recorded under voltage clamp as the synchronous activation of around 1500 glycine "channels". They were unable to record IPSCs when tetrodotoxin was included in the bathing solution: since this drug blocks nervous conduction, the result suggests that the spontaneous potentials seen in the present experiments were related to activity in presynaptic terminals rather than to spontaneous quantal release of transmitter.

Gage & Hamill (1976, 1981) have examined the effects of several anaesthetics on motor end plate currents and miniature end plate potentials in the mouse diaphragm preparation, and pointed out certain differences between drugs. Barbiturates speeded the decay of end plate currents and so limited the amplitude of the end plate potential (EPP). This action was shared by several volatile anaesthetics to an extent which was correlated with their lipid solubilities. Despite reducing the end plate channel lifetime, high concentrations of ether increased the duration of the EPP; a result attributed to an anticholinesterase action of ether. The short chain alcohols such as ethanol potentiated neuromuscular transmission by increasing the lifetime of acetylcholine channels. The authors concluded that there was insufficient evidence to support any particular molecular model of the anaesthetic effects.

Anaesthetics have long been known to disrupt brain function both by inhibiting conduction of the nervous impulse and, at lower concentrations, by interfering with inter-neuronal communication (Larrabee & Posternak, 1952). The result must be to distort coded information by altering the pattern of impulse traffic. If the activities of the bulbar cells or of their inputs are appreciably affected by anaesthetic drugs, this should be reflected in the recorded spontaneous activity. It is of particular interest to investigate the activity of reticular cells because the reticular activating system has been implicated in the control of wakefulness and has therefore been considered to be a prime target for anaesthetic actions (Moruzzi & Magoun, 1949). The bulbar Müller cells, however, are not directly concerned with mechanisms of consciousness, rather do they contribute to somatic motor control. Anaesthetics are known to have dissimilar effects on the EEG recorded from the reticular formation (Winters et al, 1972; Figure IV-6 and see Section I). Such differences may be reflected in differential effects on the synaptic potentials recorded from single neurones, and may be caused by a combination of pre- and post-synaptic actions. Full discussion of the results of this section has been deferred until Section VII so that the results of the experiments in which postsynaptic effects were examined in isolation can be taken into account.

Methods

The procedures for dissection and making electrophysiological recordings from bulbar Müller cells have been described in Section

II. After a cell had been penetrated, a continuous record of its membrane potential was made using the pen recorder at high gain and with a slow paper speed. The shapes of individual potential events could more readily be seen on the oscilloscope screen. Detailed analysis of individual potentials was not envisaged so a photographic record was not routinely made.

The preparation was perfused with a Ringer solution containing a physiological concentration of magnesium (1.8mM: Solution C). Most experiments were conducted at 10°C, but the spontaneous activity was originally noted during perfusion with Ringer at room temperature (water temperature 15-18°C). The effect on the activity of lowering the temperature to 2°C was also noted. Sometimes the same cells were later used for iontophoresis experiments. In these cases the $[Mg^{2+}]$ was increased (perfusate changed to Solution D) while recording continued to check that the spontaneous potentials were indeed blocked as a result.

The results are presented as chart records in which a potential "event" appears as a transient vertical displacement of the trace. The lateral separation of the transients is indicative of the frequency of events; transient length is a measure of the amplitude of the potential displacements, within the limits imposed by the frequency response of the chart recorder. The duration of a typical small EPSP was 40ms: if this be regarded as the wavelength of the fundamental signal, the implied frequency is 25Hz. Faithful reproduction of the signal would therefore require a flat response up to frequencies of the order of 250Hz. The manufacturer's specification states that the frequency response of the chart

recorder is flat ±0.5dB at 20mm p-p deflection to 100Hz, and flat ±0.5dB at 50mm p-p deflection to better than 50Hz. It is therefore likely that the shapes particularly of the larger potentials were distorted - at the gain used routinely, small EPSPs caused pen excursions of 8-12mm. Had a quantitative analysis been envisaged, a different recording technique would have been appropriate, but for the purposes of this study neither this source of distortion nor the tangent distortion introduced by the design of the pen recorder was important.

Anaesthetics were applied dissolved in the perfusing Ringer. No osmotic compensation was made because the anaesthetic concentration never exceeded 10^{-3} M. When repeated applications were made to a preparation, at least twenty minutes was allowed as a recovery period between tests. More usually this period was between thirty and forty minutes; but when successive solutions were applied in order of increasing anaesthetic concentration there was sometimes no intervening wash.

Results

Spontaneous synaptic potentials

When intracellular potential recordings were made at high gain, small brief perturbations of the membrane potential could be distinguished. Such spontaneous potential changes occurred in both hyperpolarising and depolarising directions, with amplitudes usually in the range 0.5 to 3.0mV. They have been interpreted as IPSPs and EPSPs respectively. The rise and decay times varied with the amplitudes of the potentials, but small events commonly lasted 40ms

and had time constants close to 10ms (rise) and 15ms (decay). When displayed on an expanded time scale so that their shapes could be seen it was evident that some of the larger potentials were composed of superimposed smaller events. There were discrete steps in the amplitudes of the potentials but, because electrical interference was appreciable when recordings were made at high gain, it was not possible to measure the smallest displacements sufficiently accurately to justify a quantitative analysis. The situation is more complex than at the neuromuscular junction where all the recorded MEPPs occur at a single synapse: there is no reason to suppose that all EPSPs recorded from a Müller cell reflected events at a single excitatory synapse. It is likely that, if the potentials were quantal in nature, the unit response at each synapse was different. This would complicate any analysis of potentials using the Poisson or binomial distributions and necessitate the accurate measurement of amplitudes. To facilitate description of the sample traces displayed in Figures IV-1 to IV-5, for each experimental condition the width of the envelope of the membrane potential trace was measured at ten equally spaced points; and the mean and standard error of each set of ten measurements was calculated.

These potential transients were likely to result from the action of small quantities of transmitter at synapses in the vicinity of the recording electrode. Their amplitude should reflect the change in conductance of the cell membrane, and the driving force (due to the electro-chemical gradient) on the ion species to which the membrane permeability was temporarily altered. However,

no change in amplitudes or polarities of the transients were detected when the resting membrane potential fluctuated (as in Figure IV-1A) - since the reversal potential for IPSPs has been shown to be only about 6mV negative to the resting potential in these cells (Wickelgren, 1977) their amplitudes would be expected to be very sensitive to membrane potential. The frequency of occurrence of spontaneous potentials showed strong temperature dependence; individual events could not be resolved at 15°C, whereas at 4°C the transients were widely spaced in time (not illustrated). Elevation of the magnesium content of the Ringer (changing from solution C to solution D) abolished all these potentials.

When the spontaneous activity was greatly increased, as during the application of glutamate or substance P, action potentials were elicited (see Section VI). A few cells showed "bursting" activity in which periodically there was a depolarisation of 6-7mV accompanied by a burst of action potentials (e.g. Figure IV-3). It is interesting that similar behaviour was also elicited by bath application of substance P (Section VI). Other cells showed intermittent hyperactivity, and it was for this reason that a continuous record of membrane potential was made although only small sections can be displayed. The records were made at slow speeds because alterations in the frequency, or in the balance between IPSPs and EPSPs, were then clearer.

The effects of anaesthetics

Each anaesthetic was tested on several preparations, initially at room temperature. Under these conditions it proved impossible to detect any influence of the anaesthetics, but a small change in

activity would not have been apparent. A further problem was the rapid deterioration of many cells. The results illustrated in Figures IV-1 to IV-5 and tabulated in Tables IV-1 to IV-4 were all taken from experiments in which the temperature in the chamber was controlled at 10°C. No permanent record of the potentials was kept in the other experiments.

Since the results have not been treated quantitatively, the Tables show only the direction of changes in the frequency of potentials: an increase in the proportion of large transients has been recorded as an increase in frequency because superimposition of small potentials had been observed. No amplitude measurements have been recorded. In a few cases there was a biphasic anaesthetic effect: for example, alphaxalone (2.7×10^{-5} M) increased the frequency before suppressing it (Figure IV-4). Such responses figure twice in the table: as "+" and as "(-)".

The IPSPs were the more susceptible to anaesthetic blockade: whereas the frequency of EPSPs was sometimes maintained or increased while that of IPSPs was reduced, the converse never occurred. If EPSPs were abolished the cell became quiescent. Similarly, during the recovery period following exposure to an anaesthetic, it was always the EPSPs which were restored first and sometimes they, alone, returned. When activity was enhanced it was always predominantly excitatory; although when the increase was pronounced, as in the presence of 3.7×10^{-3} M ketamine (Figure IV-6), it was impossible to resolve individual events and to ascribe a polarity to them.

Most cells had resting membrane potentials in the range -60 to -70mV. No anaesthetic consistently altered the membrane potential. Measurements of input resistance were not made in all cells. Those that were made, and those made during the iontophoresis experiments, showed that the highest concentrations of pentobarbitone and metomidate (10^{-3} M), only, affected the input resistance, causing a small reduction.

Pentobarbitone

Pentobarbitone was tested at different concentrations on a total of 8 cells from 5 preparations. Of these, results from three cells in a single preparation are tabulated (Table IV-1), and typical records are displayed in Figure IV-1.

There was a clear reversible depressant effect on all the cells tested. It was a consistent finding that IPSPs were the first lost; and that they reappeared, if at all, later than the EPSPs when the preparation was washed. Increasing the concentration of pentobarbitone from 10^{-4}M to 10^{-3} M served only to emphasise the depression. In one experiment from which sample records are shown, the width of the envelope was measured in each experimental condition; the figures (mean ±s.e.m., mV; n=10) were:

Cell A:	Control	1.36 ±0.149
	Pentobarbitone 2.5×10^{-4} M	0.90 ±0.080
	Wash	0.78 ±0.039
	" (later)	1.15 ±0.106

In this cell pentobarbitone reversibly depressed the mean width

Figure IV-1

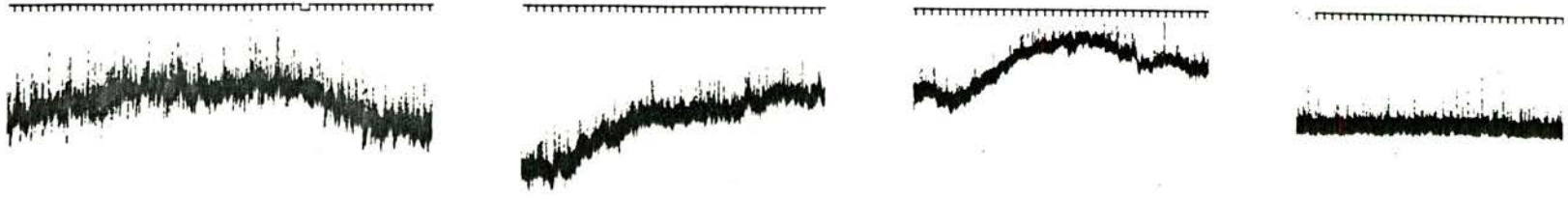
The effects of pentobarbitone on spontaneous synaptic activity

(A) and (B) are sample records from different preparations. Note that in both cases hyperpolarising potentials (downward transients) are more completely lost and return later (B) or not at all (A). Note also, particularly in (A), the apparent absence of effect of fluctuations in membrane potential on the amplitudes and polarities of the transients.

Exposure times: (A) 2.5×10^{-4} M 5min; 10^{-3} M 2min

(B) 2.5×10^{-4} M 4min

Wash time: (A) 22min



Control

$2 \cdot 5 \times 10^{-4}$ M

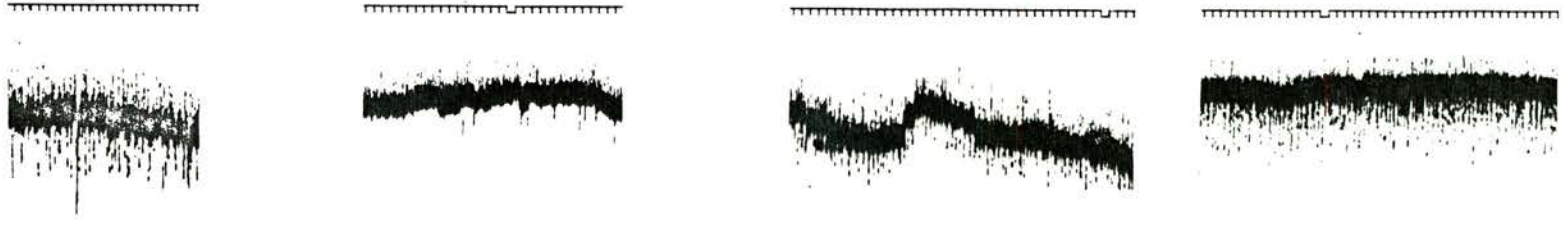
10^{-3} M

Wash

Pentobarbitone

1 mV

4 s



Control

$2 \cdot 5 \times 10^{-4}$ M

(10 min)

(15 min)

Pentobarbitone

Wash

Table IV-1

The effect of pentobarbitone on spontaneous synaptic activity

	IPSPs			EPSPs		
Pentobarb. (M)	+	-	n.c.	+	-	n.c.
10^{-4}	-	1	-	-	1	-
3-5 x 10^{-4}	-	2	-	-	2	-
10^{-3}	-	1	-	-	1	-

Figures tabulated are the numbers of tests in which an increased (+), decreased (-) or unchanged (n.c.) level of activity (frequency of potential transients) was observed.

of the envelope by 34%. The method is not entirely satisfactory because it fails to distinguish between EPSPs and IPSPs. The mean value gives some indication of the amplitudes of potential transients, whereas the standard error is related more directly to their frequency of occurrence: small values for the latter would be expected when transients were either very frequent or very rare.

Ketamine

Ketamine at various concentrations was tested on a total of 15 cells from 11 preparations. Experiments conducted at room temperature were hard to interpret, but Figures IV-2 and IV-3 show sample records from a preparation tested at 10°C: the three cells tested in this preparation all responded similarly. The collected results from cells tested at 10°C are tabulated (Table IV-2).

In the cell illustrated, changes effected by ketamine at concentrations below 10^{-4} M were not dramatic, with the exception of the intermittent bursting activity recorded in the presence of 3.7×10^{-7} M ketamine. This was an isolated observation although a dramatic result, and it cannot be certain that it was a genuine effect of the drug. However, increased activity was also noted in the presence of 3.7×10^{-6} M ketamine, and was a transient observation during the onset of action of more concentrated solutions.

The depressant effects of 3.7×10^{-4} M ketamine were confined to those on the frequency of transients, the amplitudes were not detectably reduced, and were common to both IPSPs and EPSPs. They were reversible on washing. In this cell the effects of the low

Figure IV-2

The effects of ketamine on spontaneous synaptic activity (part 1)

Sample records taken from 1 experiment. Depolarising potentials (upward transients) increased by 3.7×10^{-5} M ketamine. This change was only partially reversed, and hyperpolarising activity appeared during the wash period. Loss of hyperpolarising transients when 3.7×10^{-4} M solution applied may be coincidental, but depolarising transients clearly reversibly reduced in amplitude and frequency.

Exposure time: 3.7×10^{-5} M 6min

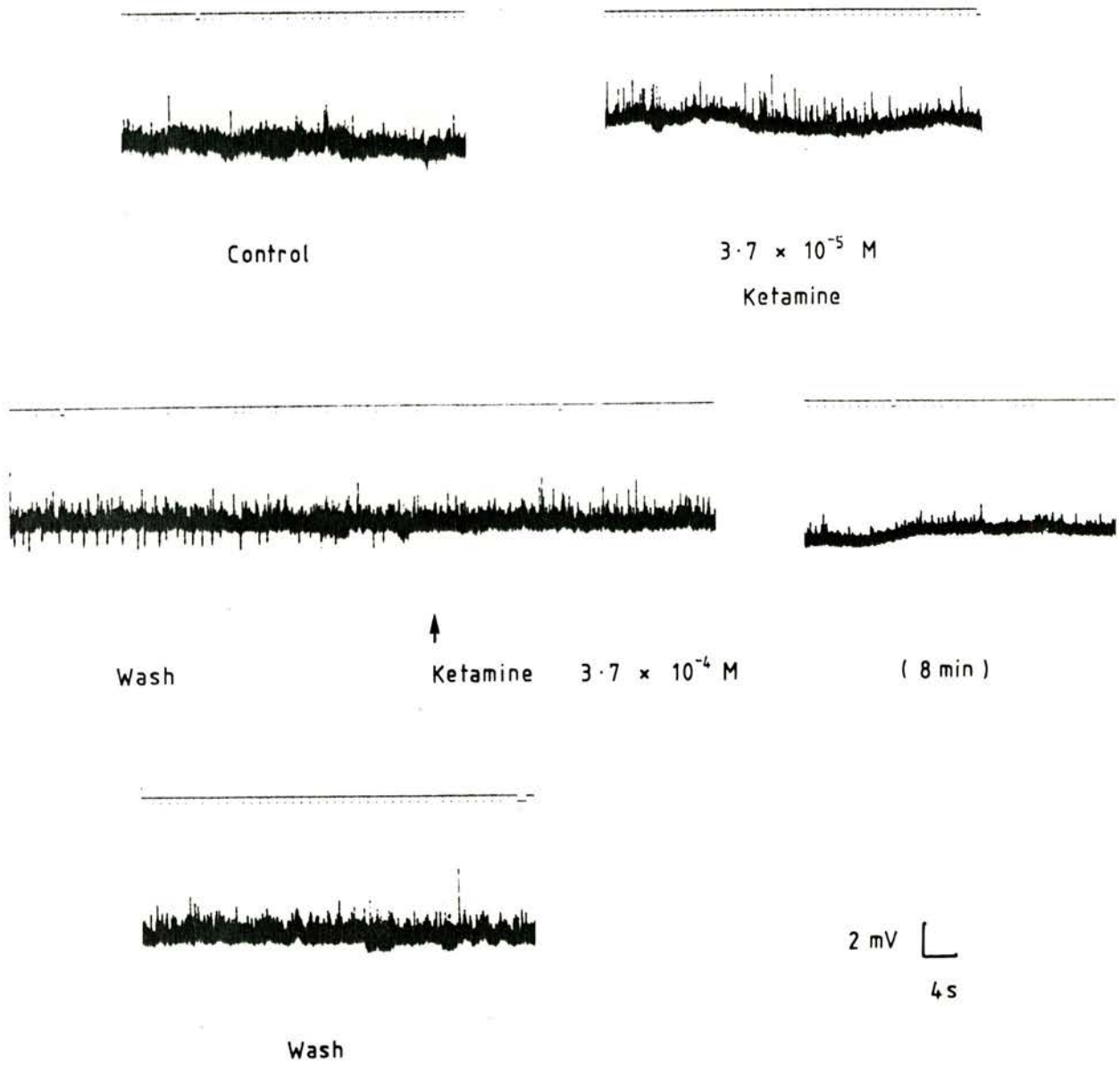

Control
3·7 × 10⁻⁵ M
Ketamine
Wash
Ketamine 3·7 × 10⁻⁴ M
(8 min)
Wash
2 mV
4 s

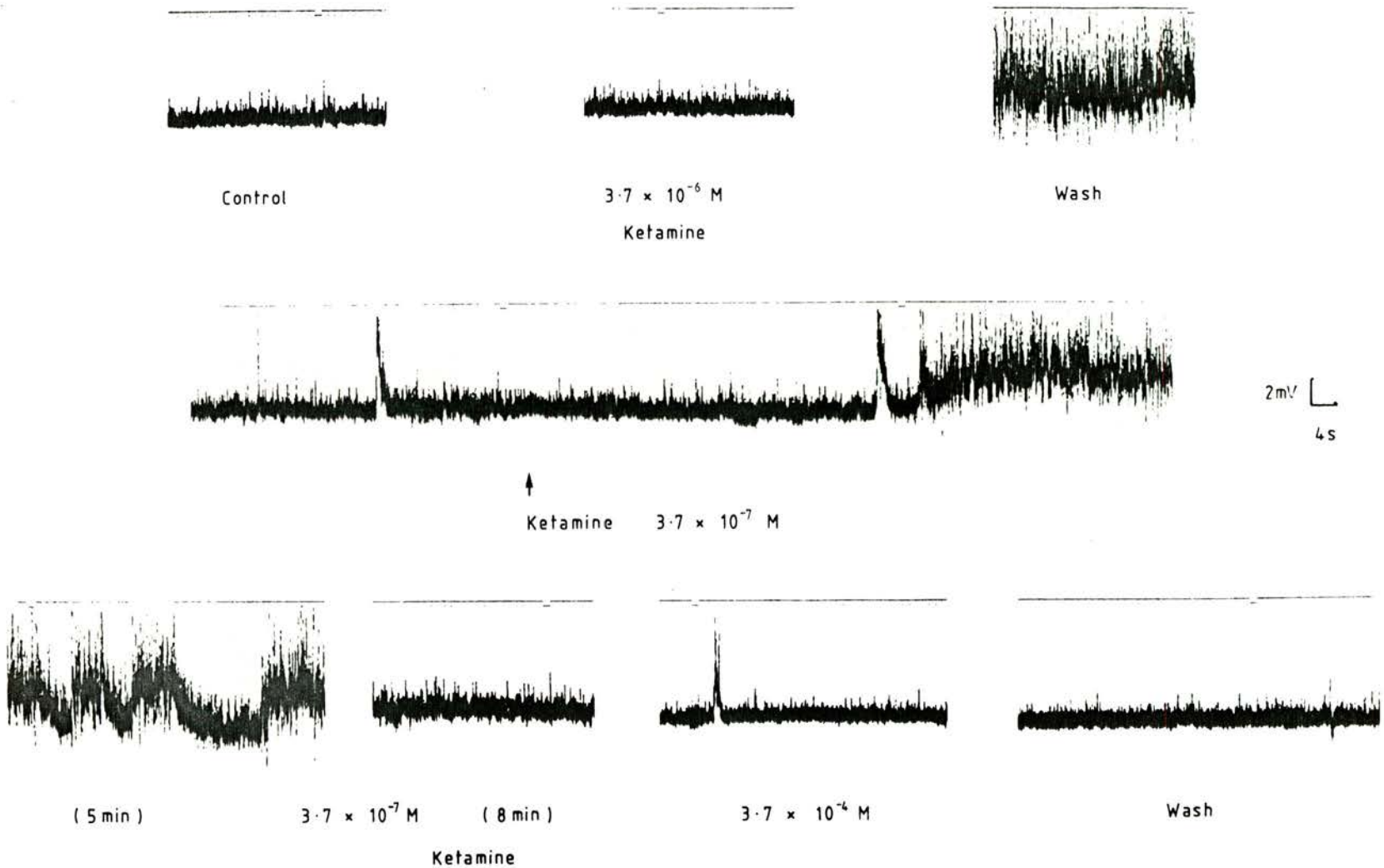
Figure IV-3

The effects of ketamine on spontaneous synaptic activity (part 2)

Records from later in the same experiment as that featured in Figure IV-2. Ketamine at 3.7×10^{-6} M had little effect, but vigorous synaptic activity developed during the wash period. This settled to intermittent depolarising excursions before ketamine (3.7×10^{-7} M) re-applied. Thereafter depolarising excursions were followed by similar vigorous synaptic activity for 1-2min. The 2 samples shown were typical of the alternating states. When the ketamine concentration was increased to 3.7×10^{-4} M smaller depolarising excursions still occurred but were not succeeded by a general increase in activity.

Exposure time: 3.7×10^{-6} M 7min

Wash time: 6.5min


Control
3·7 × 10⁻⁶ M
Ketamine
Wash
2mV
4s
Ketamine 3·7 × 10⁻⁷ M
(5 min)
3·7 × 10⁻⁷ M
(8 min)
Ketamine
3·7 × 10⁻⁴ M
Wash

concentrations (Figure IV-3) were tested after washing off a more concentrated solution (Figure IV-2), a fact which may have distorted the results if traces of ketamine remained. When a 3.7×10^{-4} M solution was reapplied following the dilute solutions, the suppression of the bursts of synaptic activity was dramatic, but the depolarising excursions of the base-line on which this activity had been superimposed remained.

The envelope measurements (mean ±s.e.m., mV; n=10) for this cell were:

Control		1.16 ±0.092	
Ketamine	3.7×10^{-5} M	1.11 ±0.088	(= 4% decrease in mean)
Wash		1.30 ±0.104	
Ketamine	3.7×10^{-4} M	0.73 ±0.042	(= 44% decrease)
Wash		1.10 ±0.086	
Ketamine	3.7×10^{-6} M	1.02 ±0.096	(= 7% decrease)
Wash		1.77 ±0.212	
(later)		2.48 ±0.318	
Ketamine	3.7×10^{-3} M	* 3.70 ±0.434	(= 49% increase)
		* 2.00 ±0.186	(= 19% decrease)
	3.7×10^{-4} M	1.08 ±0.044	(= 46% or 71% decrease from previous test)
Wash		1.30 ±0.096	

* these measurements were taken from samples representative of the alternating states (see Figure IV-3).

Table IV-2

The effect of ketamine on spontaneous synaptic activity.

	IPSPs			EPSPs		
Ketamine (M)	+	-	n.c.	+	-	n.c.
3.7×10^{-7}	1	-	-	1	-	-
3.7×10^{-6}	-	-	1	1	-	-
3.7×10^{-5}	-	2	3	3	-	2
3.7×10^{-4}	-	4	-	-	4	-

Figures tabulated are the numbers of tests in which an increased (+), decreased (-) or unchanged (n.c.) level of activity (frequency of potential transients) was observed.

Alphaxalone

Alphaxalone was tested on 4 cells from 4 preparations with the results tabulated in Table IV-3. The 2.7×10^{-6} M solution was tested after it had been found that the higher concentration (2.7×10^{-5} M) induced a transitory increase in activity. A clear increase in the depolarising activity was produced by the lower dose in 1 of 2 cells tested; in this cell the increase coincided with a decrease in IPSP frequency. In some cells few IPSPs could be distinguished from the baseline noise: but even in these a transient alphaxalone-induced increase in EPSP frequency could be detected (e.g. Figure IV-4B).

The late depressant effect of alphaxalone (2.7×10^{-5} M) was seen on more than one occasion (e.g. Figures IV-4A & B); and was not just the result of cell deterioration, because in one case (Figure IV-4A) a measure of reversal was obtained on washing: EPSPs but not IPSPs returned.

The envelope measurements for the cells illustrated (mean ±s.e.m., mV; n=10) were:

Cell A:	Control	1.22 ±0.062	
	Alphaxalone (2.7×10^{-5} M)	1.40 ±0.128	(17% increase)
	Wash	1.14 ±0.042	
Cell B:	Control	0.78 ±0.063	
	Alphaxalone (2.7×10^{-6} M)	0.96 ±0.106	(23% increase)
	Wash	0.86 ±0.100	
	Alphaxalone (2.7×10^{-5} M)	0.80 ±0.109	(7% decrease)
	(later)	0.53 ±0.021	(38% decrease)

Figure IV-4

The effects of alphaxalone on spontaneous synaptic activity

(A) and (B) are samples from 2 different experiments. In both, alphaxalone (2.7×10^{-5} M) transitorily increased the frequency of depolarising (upward) transients. Hyperpolarising transients were also more frequent in (A). Prolonged application of alphaxalone caused loss of activity (A): this was not always reversible (contrast A and B). Despite the early increase in activity following 2.7×10^{-5} M alphaxalone, there was (A) no clear increase when 2.7×10^{-6} M alphaxalone was applied.

Exposure times: (A) 2.7×10^{-5} M 2.5min

(B) 2.7×10^{-5} M 1.5min

Wash time: (A) 10min

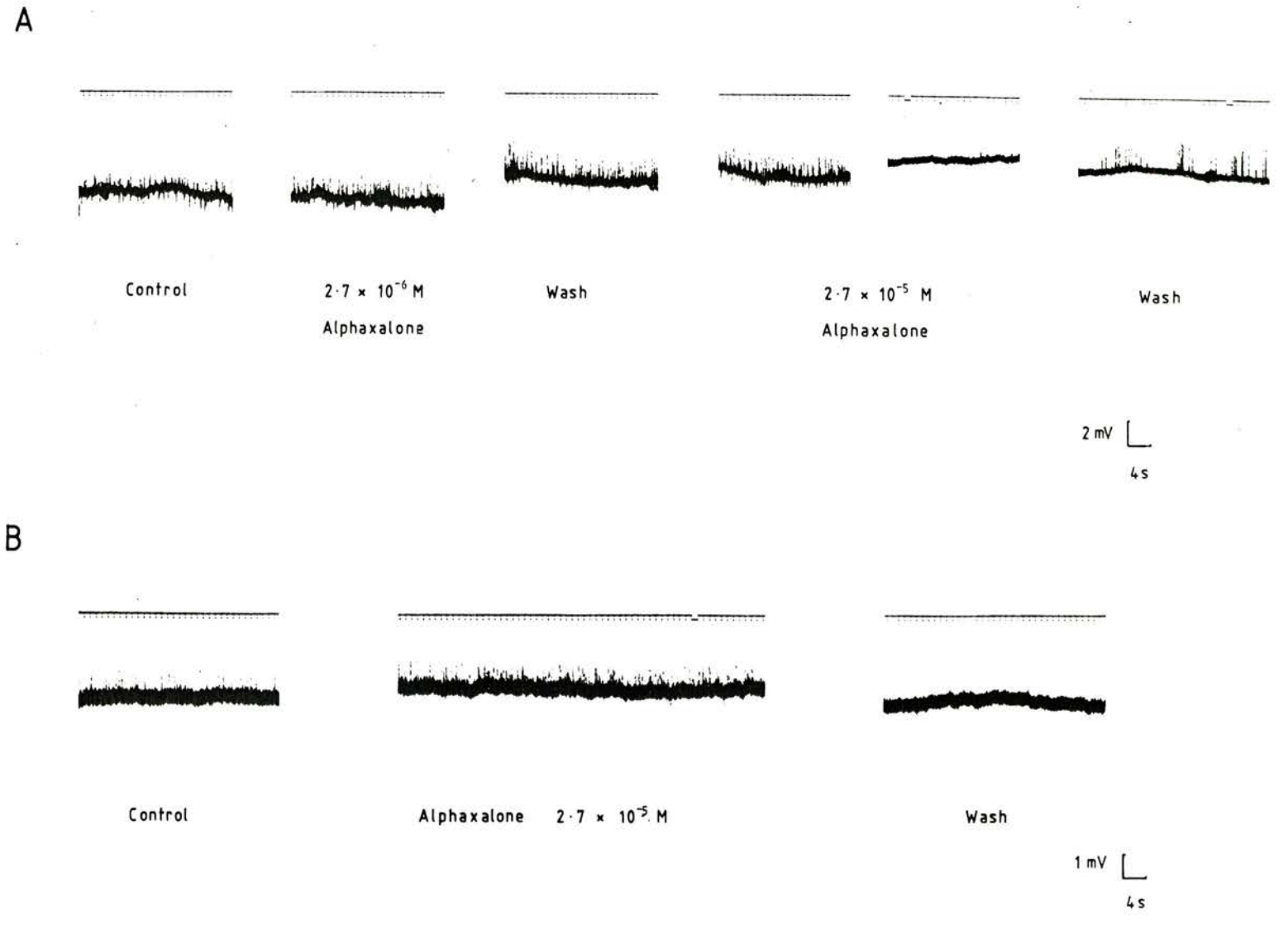

A
Control
2·7 × 10⁻⁶ M
Alphaxalone
Wash
2·7 × 10⁻⁵ M
Alphaxalone
Wash
2 mV
4 s
B
Control
Alphaxalone 2·7 × 10⁻⁵ M
Wash
1 mV
4 s

Table IV-3

The effect of alphaxalone on spontaneous synaptic activity

	IPSPs				EPSPs			
Alphaxalone (M)	+	-	n.c.	(-)	+	-	n.c.	(-)
2.7×10^{-6}	-	1	1	-	1	-	1	-
2.7×10^{-5}	1	1	4	1	4	-	2	2

Figures tabulated are the numbers of tests in which an increased (+), decreased (-) or unchanged (n.c.) level of activity (frequency of potential transients) was observed. Tests recorded in the columns headed "(-)" are those in which the activity was reduced only after first having been increased (see p.94).

Metomidate

Records were obtained of tests on 5 cells from 2 preparations exposed to different concentrations of metomidate. The results are tabulated (Table IV-4).

That concentration nearest to the clinically relevant figure (1.8×10^{-5} M) had no consistent effect on the spontaneous activity, and even with 1.8×10^{-4} M the effects were not dramatic. Depression by the high concentration (1.8×10^{-3} M) was much more striking, and once again the IPSPs were those first affected. The depression illustrated (Figure IV-5) was reversed on washing the preparation, albeit not to the previous condition.

Envelope measurements were made on the records from the cell illustrated, and were as follows: (mean ±s.e.m., mV; n=10)

Control		1.42 ±0.154	
Metomidate	(1.8×10^{-5} M)	0.90 ±0.115	(37% decrease)
	(1.8×10^{-4} M)	0.76 ±0.062	(46% decrease)*
	(1.8×10^{-3} M)	0.52 ±0.032	(63% decrease)*
Wash		0.65 ±0.065	

* these figures are the calculated percentage decrease from the control values.

Discussion

Concentration of drugs in the brain

There is a problem in deciding what concentration of an anaesthetic applied to an isolated CNS preparation best mimics conditions obtaining in the brain during surgical anaesthesia. The

Figure IV-5

The effects of metomidate on spontaneous synaptic activity

Sample records from a single experiment. Metomidate had little effect at 1.8×10^{-5} M, but in the presence of 1.8×10^{-4} M there was a shift in balance so that depolarising transients were relatively more frequent. When 1.8×10^{-3} M metomidate was applied the hyperpolarising transients were abolished completely before the depolarising activity was reduced. A measure of restoration of both types of activity was achieved by washing the preparation for 26min.

Exposure times: 1.8×10^{-5} M 5min (total 8min)

1.8×10^{-4} M 7min (total 8min)

1.8×10^{-3} M 4min (total 6min)

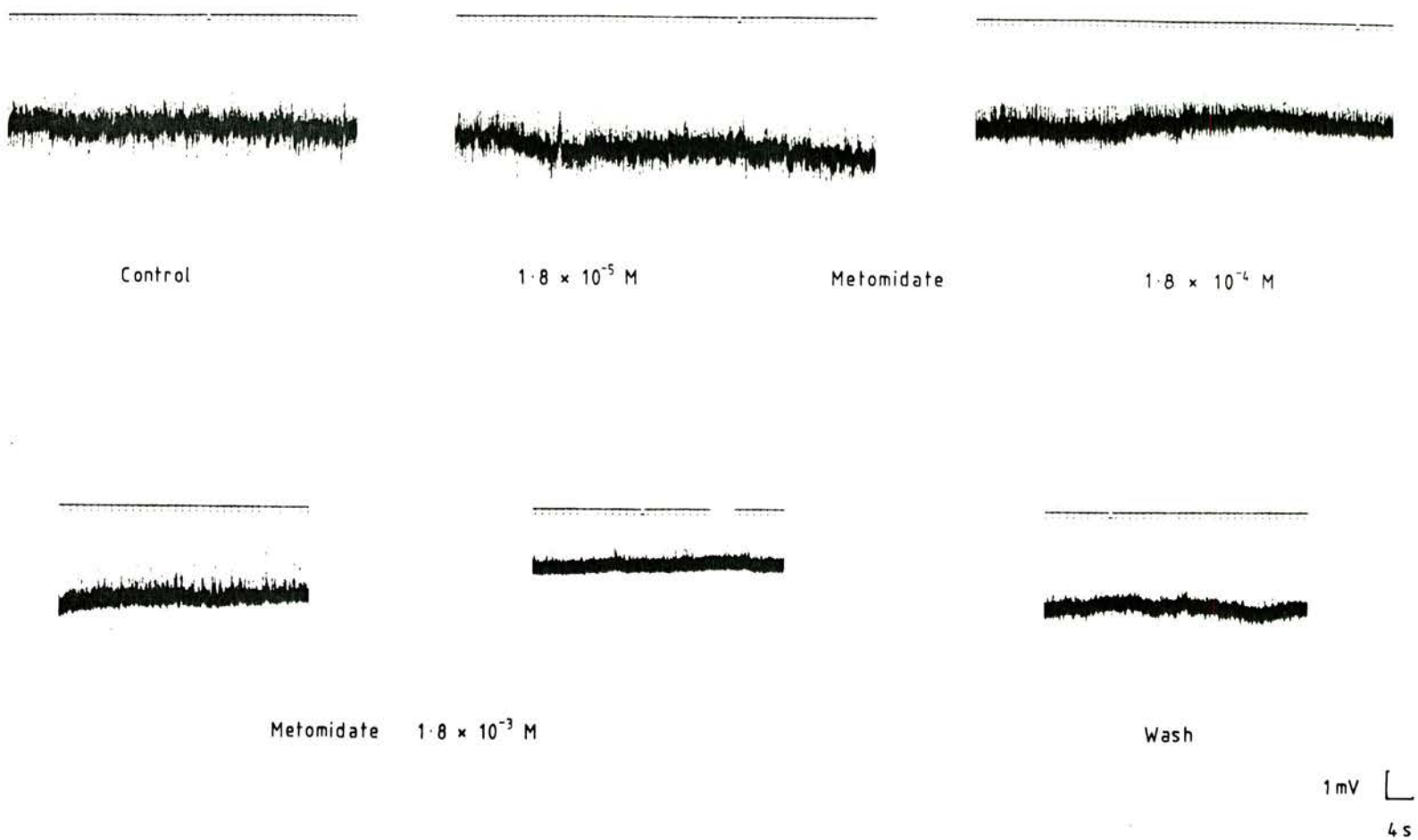

Control
1·8 × 10⁻⁵ M
Metomidate
1·8 × 10⁻⁴ M
Metomidate 1·8 × 10⁻³ M
Wash
1 mV
4 s

Table IV-4

The effect of metomidate on spontaneous synaptic activity

	IPSPs				EPSPs			
Metomidate (M)	+	-	n.c.	(-)	+	-	n.c.	(-)
1.8×10^{-5}	-	2	5	-	1	-	3	2
1.8×10^{-4}	1	2	1	-	1	2	1	-
1.8×10^{-3}	1	2	-	1	1	3	-	1

Tabulated figures are the numbers of tests in which an increased (+), decreased (-) or unchanged (n.c.) level of activity (frequency of potential transients) was observed. Tests recorded in the columns headed "(-)" are those in which the activity was reduced only after first having been increased (see p.94).

most readily available figures are the induction doses of anaesthetics in a variety of mammalian species. In the case of a gaseous or volatile anaesthetic the problem is reduced because the partial pressure of anaesthetic in the end-tidal air can be measured: this air is in equilibrium with venous blood and thence with the brain. With injectable drugs the influence of factors such as absorption from the injection site, binding to plasma proteins, the relative uptake by the brain and other tissues, and the rate of elimination cannot be ignored, but can be only estimated. It may be most appropriate to use the drug concentration found clinically in the CSF, because the preparation is likely to concentrate drugs from the Ringer in the same manner as does the brain from the blood.

Translation of this principle into the experimental situation is complicated by the absence of reliable information as to the CSF concentrations of the various anaesthetics during surgical anaesthesia. Blood concentrations are available in some cases (pentobarbitone, ketamine, etomidate), but for the rest they must be estimated with due allowance for the effect of protein binding and for the extent of distribution within the body (See Table III-5). The brain concentration of a drug will be under- or over-estimated according as the drug is preferentially distributed to the brain or is eliminated faster than it is absorbed from the site of injection. The problem was skirted by Schueler & Gross (1950) who took blood from an anaesthetised animal in order to study the effect of the anaesthetic on respiration in isolated brain slices.

Even could it be established that experiments were conducted with appropriate concentrations of drugs, the picture would not be

clear. Anaesthesia has no clearly defined end-point: the neuronal derangement is graded as are the effects of the drugs on isolated tissues. What is a barely detectable effect in an electrophysiological experiment may have either little or considerable impact on the normal functioning of the cell. It is well known to clinicians that the depth of anaesthesia is affected by the intensity of the surgical stimulus: much higher doses of anaesthetic are required to prevent motor responses to certain very painful operations, such as those on the hip or the ear. Yet the drug does not disappear from the brain when the stimulus intensity is increased.

Anaesthesia and electrical activity in the brain

In assessing the results of the experiments in this Section it must be made clear what has been measured. The spontaneous activity recorded from a bulbar Müller cell must be the response of the cell to the activity impinging on it, because abolition of synaptic transmission results in a quiescent preparation. A change in the former activity may result from changes in the receptor or other membrane properties of the penetrated cell, or from presynaptic changes originating elsewhere in the CNS - if it can validly be assumed that the spontaneous potentials are not merely the central equivalent of MEPPs at the motor end plate. No distinction can be made between these possibilities on the basis of the present experiments alone. However, the resultant change in activity of the Muller cells will have repercussions for those cells with which they make efferent synapses.

The input to the Müller cells is of many modalities (Rovainen,

1967; Wickelgren, 1977) and represents the resultant of central integrative processes, insofar as these determine the appropriate motor response in any set of circumstances. A reduction in afferent activity reflects either a reduced sensory input or a suspension of the integrative processes from whatever cause. In the present case it is not possible to state that a recorded decrease in activity was the result of anaesthetic effects on the Müller cell's receptors rather than on presynaptic processes. The techniques employed could not measure detailed changes in responses at individual synapses; nor was a statistical analysis of synaptic potentials after the manner of del Castillo & Katz (1954) feasible, although some larger potentials were seen to be composed of super-imposed smaller events. There would in any case be little benefit in analysing the potentials in detail because, as already discussed, the use of techniques enabling measurement of single channel conductance is to be preferred, and is likely to be possible in this preparation. Werman (1969) has discussed in detail the relative merits of potential and conductance measurements in pharmacological experiments: conductance changes are closer to the fundamental drug action than are potential changes, and are therefore a truer reflection of it.

The output of the Müller cells is to the ipsilateral segmental motoneurones and thence to the somatic musculature. Thus stimulation of the bulbar cells elicits a variety of movements (Rovainen, 1967; and see Section I). However, a recent study of spinal interneurones (Buchanan, 1982; Buchanan & Cohen, 1982) has shown that bulbar cell stimulation excites also those interneurones

which inhibit the contralateral motoneurones, an action which must be important in motor coordination. Reduced activity in the Müller cells is likely to disinhibit these contralateral motoneurones thereby causing uncoordinated movements when muscles on both sides of the body contract simultaneously. An effect of this type could explain at least partially the observation (Section III) that during the induction period uncoordinated movements occurred: both rolling movements and frequent waves of contraction which passed down the body yet were non-propulsive and of small amplitude.

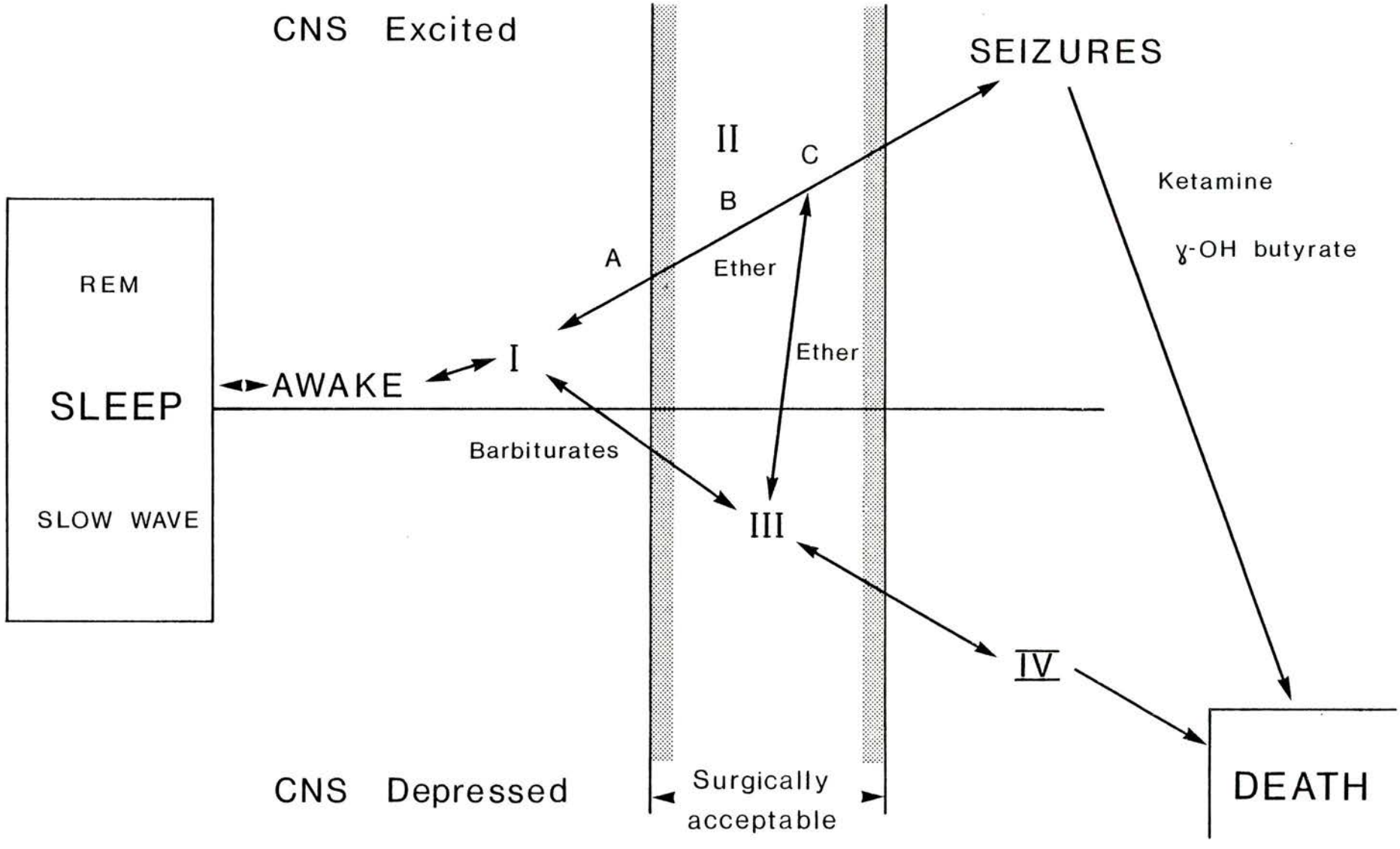
It came as no surprise to find that high concentrations of all four anaesthetics suppressed the spontaneous activity. Reference to Table III-5 shows that these were concentrations far in excess of those encountered clinically, and previous studies have indicated that the selectivity factor, by which concentrations blocking synaptic transmission and axonal conduction differ, is about 10 (e.g. Larrabee & Posternak, 1952).

Consideration of the effects of low concentrations of anaesthetics is of more interest. Until recently, anaesthesia was equated with a reduction in the electrical activity of the brain (e.g. Bernard, 1875) that proceded ultimately to the electrical silence of death. Many of the early EEG studies were performed using the anaesthetics ether and pentobarbitone, a choice which was both fortuitous and misleading since both drugs, in concentrations producing a surgically acceptable state, do indeed depress the activity. Winters and his co-workers (Winters et al, 1967; Winters et al, 1972) have since shown that, far from being depressed, the EEG is excited by some of the other barbiturates and

Figure IV-6

Diagram to show the multiple states of anaesthesia
(Adapted from Winters et al, 1972)

All anaesthetics induce the initial excitement (Stage I). Thereafter some (e.g. barbiturates) induce surgical anaesthesia directly; whereas others (e.g. ether) produce an intermediate stage (Stage II) of increased CNS excitement, manifested as hallucinations (A,B) and catalepsy (C). Drugs such as ketamine, phencyclidine and γ-hydroxybuyrate do not depress the CNS. The righting reflex is lost during Stage IIC. Stages IIC and III are surgically acceptable because motor responses are absent and the patient is amnesic. The further stages, myoclonic jerking/convulsions and Stage IV (medullary paralysis) are unacceptable although usually reversible.
REM: "rapid eye movement" or "dream" sleep.


CNS Excited
SEIZURES
II
C
B
A
Ether
Ketamine
γ-OH butyrate
REM
SLEEP
SLOW WAVE
AWAKE
I
Ether
Barbiturates
III
IV
CNS Depressed
Surgically acceptable
DEATH

by drugs such as γ-hydroxybutyrate, phencyclidine and ketamine; these latter producing EEG changes akin to those of REM sleep, when the brain is highly alert. Winters proceded to classify anew the progressive CNS changes produced by anaesthetics and convulsants: Figure IV-6 indicates that some compounds commonly regarded as anaesthetics never produce CNS depression, the excitatory changes progressing instead through convulsions to death. Of the drugs used in this study, pentobarbitone is a conventional depressant whereas ketamine does not elicit the depressed stages (III and IV) but has epileptoid activity. The changes induced by alphaxalone and etomidate have been likened to those of γ-hydroxybutyrate and anaesthetic barbiturates respectively.

In view of this it was interesting to see whether "convulsant" anaesthetics produced an increase in the afferent input to the bulbar Müller cells at concentrations below those likely to block axonal conduction. Depolarising activity was sometimes increased by "clinical" concentrations of ketamine, alphaxalone and metomidate, but invariably reduced by pentobarbitone. However, the changes were time-dependent and increased activity was usually seen only in the first few minutes after application of the drug was commenced, presumably as the concentration built up at the sites of action. The absence of a steady state effect, other than one of total depression by high concentrations, raises the problem of how to "freeze" what is essentially a continuous process, and is one reason why these results should be regarded as qualitative rather than quantitative.

Observations on the hyperpolarising potentials were less certain since these were generally more labile (e.g. their abrupt appearance and disappearance in Figure IV-2, which may or may not have related to the effects of ketamine). IPSPs were not detected in all cells, but when present they were consistently more readily depressed by anaesthetics than were EPSPs. It is interesting that IPSPs are produced by stimulation of the ipsilateral vestibular nerve (Wickelgren, 1977). Whatever the neuronal mechanism of responses to vestibular stimulation in the lamprey, it is likely that the early loss of inhibitory potentials in the Müller cells is related to the fact that loss of balance is an early sequel to anaesthetic administration. Such loss may have contributed to the rolling reported in Section III; and in higher animals is an effect caused by all anaesthetics, hence the widespread use of the loss of the righting reflex as a test for anaesthesia. This test does no more than assess the integrity of one reflex pathway, and is not relevant to the level of consciousness.

SECTION V

Anaesthetics and Inhibitory Transmitters

Introduction

Amino acid transmitters in the CNS

For many years after their presence in brain tissue was first recognised, and after inhibitory effects were demonstrated, neither GABA nor glycine was considered to be a good candidate for the role of inhibitory central neurotransmitter. GABA was identified in brain homogenates in 1950 (Awapara et al, 1950; Roberts & Frankel, 1950; Udenfriend, 1950) and was subsequently found to be depressant when applied to the cerebral cortex (Hayashi, 1952; Iwama & Jasper, 1957; Purpura, Girado & Grundfest, 1957), spinal cord (Curtis, Phillis & Watkins, 1959) and cerebellum (Purpura et al, 1957). Indeed, Krnjević & Phillis (1963) found that GABA depressed the firing of all cells to which it was applied in the cerebellum and cerebral cortex.

Glycine was identified in the CNS simultaneously (Roberts & Frankel, 1950) and was assumed to have the same properties, since the structures of the two compounds were so similar (Curtis & Watkins, 1960). Investigation of the effects of exogenous GABA showed that it inhibited both the EPSPs and IPSPs of spinal motoneurones and increased their membrane conductance. It did not however produce the hyperpolarisation characteristic of the natural inhibitory transmitter released in response to stimulation of a peripheral nerve (Curtis, Phillis & Watkins, 1959), and was not inhibited by strychnine, as was the natural inhibitory system. GABA was therefore rejected as a CNS inhibitory transmitter, and with it glycine, although the latter had received only perfunctory attention.

Recognition that although the two substances acted similarly (Krnjević & Schwartz, 1966,1967; Werman, Davidoff & Aprison, 1968) they did so at specific sites which did not show cross-sensitivity, and were to a large extent differently distributed (glycine mostly in the spinal cord and GABA in the brain) led to a reexamination of the transmitter status of both compounds. Transmitter functions were formally proposed for GABA by Krnjević & Schwartz (1966), and for glycine by Davidoff et al (1967). Glycine was thought to be the inhibitory transmitter in the spinal cord because its distribution there corresponded to that of known inhibitory synapses, and because parallel functional losses and depletions both of neurones and of glycine occurred following experimental anaemic anoxia of the cord, whereas there was no equivalent change in GABA levels. GABA exerts presynaptic inhibitory effects on the central terminals of primary afferents, which accounts in part for the early confusion over its relationship with the natural post-synaptic inhibitory pathways.

Experimental studies of inhibitory amino acids have since mushroomed and it is now generally accepted that glycine is a major transmitter in the spinal cord and lower parts of the brainstem, whereas GABA fulfils this role in the cerebral cortex. It is not immediately clear what are the functional roles of GABA receptors in those other parts of the nervous system where the receptors are apparently extrasynaptic. For instance, in the dorsal root ganglion (DRG) GABA exerts a potent depolarising effect (Nishi, Minota & Karczmar, 1974; Desarmenien, Feltz & Headley, 1980) but there are no afferent synapses, and in autonomic ganglia a similar position

obtains (de Groat, 1970; Bowery & Brown, 1974). In both sites exogenous glycine had no comparable effect, and the GABA effects, although depolarising, were chloride sensitive. This implied that tissues differ in their chloride content - Coombs, Eccles & Fatt, (1955) had shown previously that the hyperpolarising IPSP in spinal motoneurones was determined mainly by the transmembrane concentration gradients and membrane conductances to chloride and potassium ions.

The work was greatly aided by the discovery that the known convulsants strychnine and picrotoxin (and later, bicuculline) acted as specific antagonists for glycine and GABA respectively: a realisation which implied that the evidence suggestive of a transmitter role for the amino acids was a good deal older. Owen & Sherrington (1911) had described strychnine reversal of the reflex inhibitory effects of peripheral nerve stimulation, but had been unable to deduce a mechanism which had adequate experimental support.

Presence of the suspected transmitter in the appropriate neurones, and identity of action with the effects of natural stimulation are but two of the criteria which must be satisfied if a transmitter function is to be accepted. Description of specific, high affinity sodium-dependent uptake mechanisms for GABA and glycine fulfilled another. For example, Iversen & Bloom (1972) found that one or other amino acid was taken up by 51% of identified synaptosomes prepared from spinal cord homogenates. Separate, similar sized populations of neurones were involved. This work suggested that GABA and glycine are quantitatively much more

important inhibitory transmitters than are those monoamine transmitters for which an inhibitory effect has been described, such as nor-adrenaline and 5-HT in the spinal cord (Engberg & Ryall, 1966; see discussion by Snyder, 1975). However, the picture is complicated because amino acids also have a role in normal cellular metabolism.

Aspects of the more recent work on GABA and glycine have been reviewed at frequent intervals (e.g. Hebb, 1970; Curtis & Johnston, 1974; Krnjević, 1974; Snyder, 1975; Roberts, Chase & Tower, 1976; Nistri & Constanti, 1979). In relation to the extrasynaptic GABA receptors, it is interesting that uptake of GABA by astrocytes, neuroglia and other equivalent satellite cells in the autonomic nervous system has been demonstrated; and that the release of GABA from glial cells is stimulated by depolarisation. Such release could be evoked were there to be a local extracellular accumulation of potassium.

Effects of GABA and Glycine

GABA and glycine exert similar effects when they are applied to a cell possessing receptors for both of them. The response consists of an increase in membrane conductance to chloride ions (e.g Barker & Ransom, 1978a). The associated change in the membrane potential varies with the tissue under test and the experimental conditions, and has repeatedly been shown to be very sensitive to the trans-membrane concentration gradient for chloride. It must therefore be assumed either that the internal chloride concentration in cells from different tissues are not identical or that the ion specificities of the "channels" operated by the transmitters differ

between tissues. A few preparations have been developed which permit closer examination of the mechanisms involved using techniques, such as noise analysis, which have previously been applied only at cholinergic synapses. Barker, McBurney & MacDonald (1982), applying current fluctuation analysis to cultured cells, have made some distinction between the chloride channels opened as a result of GABA and glycine's binding to their respective receptors. In particular, the estimated lifetime and unit conductance of channels activated by the two amino acids on an individual cell differed, as did the relative charge transfer associated with the channels (GABA:glycine 1.00:0.74). However, Barker et al were unable to conclude that the two transmitters must be operating distinct channels, because it remained possible that the different configurational changes in the channel protein necessarily produced on binding the transmitters would result in different permeabilities to chloride ions.

The recent reports of Gold & Martin (1982a,b) concerning glycine responses indicate that techniques of noise analysis can be applied to the bulbar Müller cells of the lamprey. Patch clamping has yet to be tried and neither technique has yet been used in experiments with anaesthetics in this preparation. In the latter technique a small patch of the surface membrane is isolated, where the recording electrode seals to it, and rectangular current pulses caused by the opening of single ion channels are measured with a high gain current-voltage converter.

Recent studies have identified multiple types of GABA and glycine receptors, particularly in invertebrates (see Andrews &

Johnston, 1979; Nistri & Constanti, 1979), and complex potential changes have sometimes been recorded especially when large doses of transmitter have been applied. Barker & Ransom (1978a) attributed the the biphasic hyperpolarising/depolarising responses they recorded in cultured spinal cells to a redistribution of chloride which altered the "driving force" on chloride ions during a long pulse. It was not explicable as desensitisation, although the latter has been demonstrated in the DRG (Desarmenien, Feltz & Headley, 1980). Regional differences in the effect of GABA according to whether it was applied to the soma or the dendrites were recorded in hippocampal pyramidal cells by two groups (Alger & Nicoll, 1979,1982; Andersen et al, 1980). Whilst it was argued that the dendritic depolarisation could be indirect, resulting from an effect on adjacent cells, Diamond (1968) had previously reported that both the somata and dendrites of goldfish Mauthner cells were sensitive to GABA, and Barker & Ransom (1978a) in their well-controlled study also recorded dendritic responses, this time hyperpolarising. There is no necessity that different GABA and glycine receptors should operate identical membrane ion channels.

Pharmacological distinctions, also, have been made between GABA receptors from different cells. GABA is able to exist in several stable configurations but some of its active analogues are more rigid and are not equally effective in all preparations. Similarly, not all GABA receptors are sensitive to the antagonists bicuculline and picrotoxin. Andrews & Johnston (1979) have suggested that GABA in its extended form interacts with receptors that are sensitive to bicuculline; whereas receptors for the folded form are insensitive.

Nistri & Constanti (1979) proposed the following grouping of GABA receptors found in the mammalian CNS:

i) sympathetic ganglia; spinal interneurones; spinal motoneurones;

ii) DRG; primary afferent terminals; cortical neurones.

Of these, the first group was relatively sensitive to bicuculline and relatively insensitive to certain key agonists such as γ-amino-β-hydroxybutyric acid. Picrotoxin was a more effective antagonist than bicuculline at GABA receptors in the second group.

A third type of receptor has now been proposed to mediate the presynaptic actions of GABA at peripheral autonomic synapses (Bowery & Hudson, 1979; Brown & Higgins, 1979; Bowery et al, 1979). This receptor is resistant to bicuculline and is stimulated by the GABA analogue baclofen to reduce transmitter release. In studies on cultured embryonic sensory neurones Dunlap (1981) showed that baclofen acted like GABA in attenuating the calcium action potential, but that it did not reduce the membrane resistance. Should a similar reduction of calcium movements occur at nerve terminals transmitter release would be inhibited. Baclofen was already known to have presynaptic effects, increasing the release of GABA and reducing that of glutamate and aspartate (see Andrews & Johnston, 1979), but these had not previously been identified with a GABA-mimetic effect.

Anaesthetics and inhibitory transmission

Anaesthesia is thought to result from a reversible drug-induced disruption of brain function. Since Sherrington (1906) drew attention to the susceptibility of reflex arcs, attention has focussed on the synapse as the site of action of anaesthetic drugs

(see Section I). It has become apparent that anaesthetics differ in their effects at individual synapses (Larrabee & Posternak, 1952) and that different central pathways have unequal sensitivities (Mark & Steiner, 1958). Indeed, as already indicated in Section I, some such differential activity is required to explain the characteristic clinical states which follow administration of the various drugs (Vickers, Wood-Smith & Stewart, 1981).

Anaesthetic enhancement of presynaptic and postsynaptic inhibition is well documented, the principal anaesthetics concerned being barbiturates, α-chloralose and ether (e.g. Eccles, Schmidt & Willis, 1963). However, it has become apparent that this is not true of all inhibitory pathways: for example, Frank & Ohta (1971) found that a variety of anaesthetics including pentobarbitone blocked the reticulo-spinal inhibitory pathway in decerebrate cats, whereas reticular facilitation showed greater resistance to inhibition. Interestingly this was particularly obvious with compounds known to cause excitement in intact mice.

In this Section, the abilities of the anaesthetics to interfere with the actions of GABA and glycine are compared. Although anaesthetics could equally affect synaptic transmission by a presynaptic action, in this study postsynaptic mechanisms alone have been considered: the transmitters were applied directly to the bulbar cells, and steps were taken to preclude trans-synaptic effects.

Methods

The procedures for dissection, intracellular recording and for the iontophoretic application of transmitters have been described in Section II.

After a few pilot experiments in which both anaesthetics and transmitters were applied in the perfusing Ringer, iontophoretic techniques were introduced, thereby allowing more precise timing of the transmitter pulses and shortening the required duration of an experiment. When transmitters were bath applied there were the twin uncertainties as to whether the same concentration of transmitter was achieved at the cell surface during successive tests, and whether applications were sufficiently long to cause desensitisation. The time (20-30s) required for completion of a change in bath solution was considerably greater than that for development of a response so any effect of an anaesthetic on the kinetics of the response must have been masked. With bath application it was rarely possible to obtain a full set of control and test exposures before cell deterioration supervened.

The experimental protocol and the method of analysis of the results have also already been described (Section II,p.64). When a suitable sequence of GABA and glycine pulses had been established, it was continued during and after perfusion of the preparation with a solution of the anaesthetic under test (dissolved in Ringer solution D). These experiments were carried out concurrently with those of Section VI, so the iontophoretic pulse sequence often consisted of a standard pulse of each of the three transmitters. The pulses were normally of the same amplitude (usually in the range

40 to 90nA for GABA and glycine), although that for glutamate was sometimes larger by a factor of 10, but the optimal duration was determined for each transmitter individually. The pulse lengths fell within the range 0.3 to 7.0s, and pulses were applied at intervals of 1 or 3 minutes. Changes in input resistance associated with the transmitter actions were assessed by injecting constant hyperpolarising current pulses (strength 0.5-1.0nA, frequency 1Hz) through the recording electrode during, and immediately before and after, each transmitter pulse. The timing of these injections was under manual control, and the necessary movement in the vicinity of the preparation was the cause of artefacts visible on some sample records.

When experiments were carried out to test the influence of transmitter concentration on the anaesthetic effect only one transmitter was used. Different concentrations were produced by employing a sequence of pulses of increasing durations. Because it was now impossible for spread of the iontophoretic current between barrels to cause more than one transmitter to be ejected, these experiments also acted as a check that the previous findings had not resulted from cross-contamination between barrels.

The results are presented as sample traces taken from typical experiments; and as bar charts in which the mean anaesthetic-induced changes in the GABA and glycine response amplitudes are represented by bars of appropriate length. Each bar represents a separate test but not necessarily a different cell. The numerical data from which the bar charts were constructed are available in Appendix I, together with the results of the tests of statistical significance.

The manner in which the measurements were made has been detailed in Section II.

Results

Responses to GABA and Glycine

Most cells had resting potentials in the range -60 to -65mV and input resistances >3MΩ. The usual response to iontophoretic application of either GABA or glycine was a transient hyperpolarisation accompanied by reduction in the input resistance of the cell. A small minority of cells (4 out of 92) showed depolarising responses to both amino acids. In some cells the potential change was minimal or varied between a small depolarisation and a small hyperpolarisation: the resting potential in these cases was presumably very close to the reversal potential for the ion channels opened by the transmitter.

High concentrations (increased pulse strength or duration) of either agonist elicited complex responses. Figure V-1A shows responses to three doses of glycine, the highest dose producing a triphasic hyperpolarising/depolarising/hyperpolarising response, and illustrates the common finding that complex responses did not retain their shape over a period of time. Typically, the depolarising component was the more labile. A more dramatic illustration of this is Figure V-1C where the initial brief hyperpolarisation was succeeded by a long-lasting depolarisation. The depolarising component was drastically reduced after twelve minutes (glycine pulses applied at 3min intervals) and was never restored. The appearance of the complex responses was sometimes yet more bizarre (Figure V-1B): only occasionally was this attributable to variations

Figure V-1

Variation of response to glycine with dose applied

A: responses of a cell to 80nA pulses of glycine.

Pulse durations (left to right): 5s, 2s, 3s.

Hyperpolarising pulses: 1nA

B: bizarre responses of 2 cells to glycine.

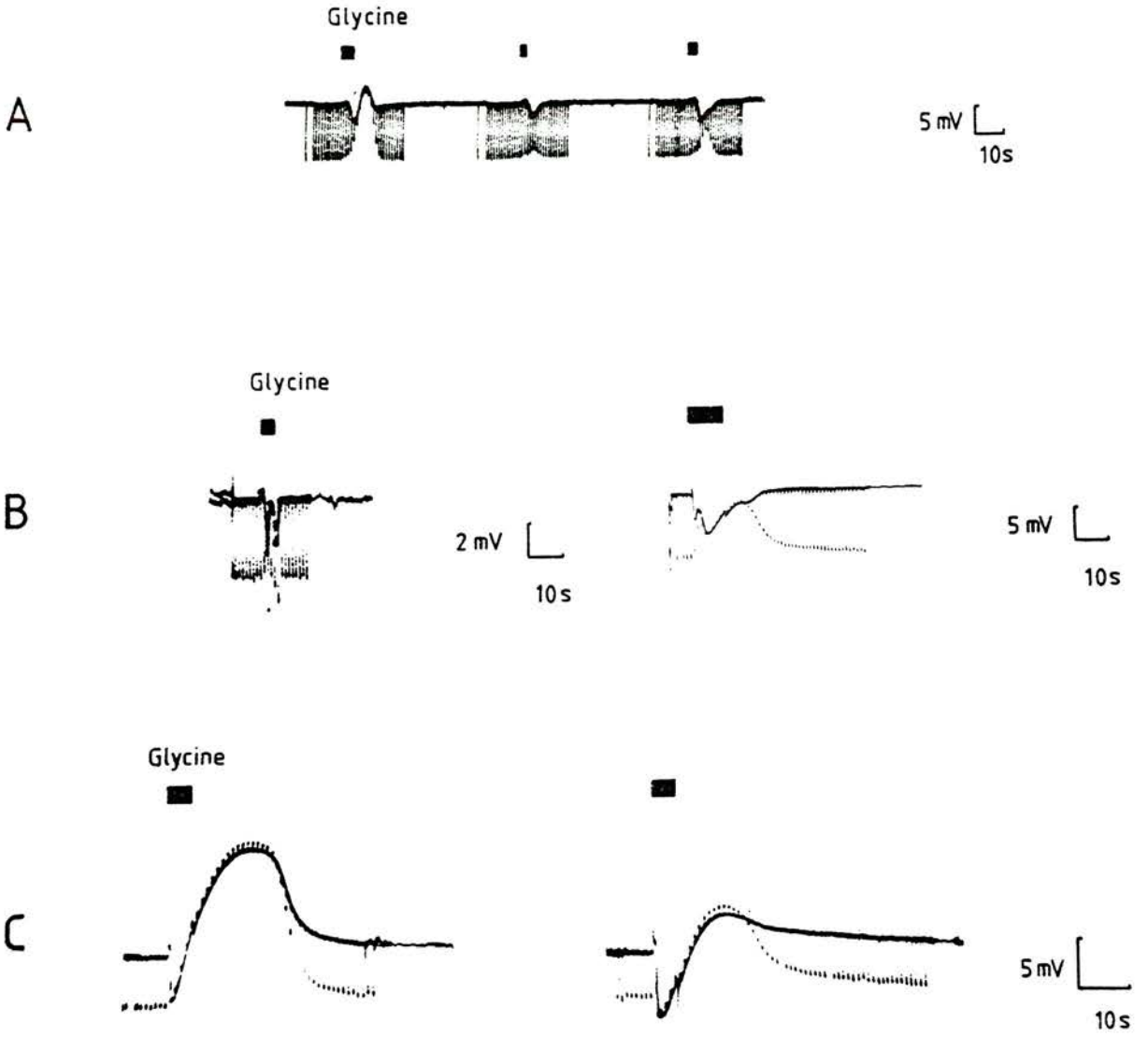
Iontophoretic pulses: (left) 200nA, 4s; (right) 300nA, 10s.

Hyperpolarising pulses: 1nA

C: desensitisation of depolarising component of complex glycine response. Glycine pulses were applied at 2 minute intervals; record on right made 12 minutes later than that on left.

Iontophoretic pulses: 200nA

Hyperpolarising pulses: 1nA


A
Glycine
5 mV
10s
B
Glycine
2 mV
10 s
5 mV
10s
C
Glycine
5 mV
10 s

in iontophoretic current. The occurrence of such responses and their changing form set an upper limit to the agonist concentrations that could be used in experiments with anaesthetics.

The effects of anaesthetics on the responses to GABA and glycine

63 ammocoetes were exposed to the anaesthetics: pentobarbitone (19 ammocoetes), ketamine (15), alphaxalone (19), metomidate (10). 10 ammocoetes were tested with the Saffan vehicle alone. No anaesthetic consistently altered the membrane potential and only the highest concentrations of pentobarbitone and metomidate (10^{-3} M) affected the input resistance, causing a small reduction. The alterations in the GABA and glycine responses measured during exposure to the four anaesthetics are displayed in Figures V-2 to V-7.

Most experiments were carried out at 8-10°C but the results were qualitatively similar at room temperature and at 2°C (not illustrated).

Pentobarbitone

The effects of pentobarbitone were complex and dose-dependent. Records from typical experiments are displayed in Figure V-2. Low concentrations (1-5 x 10^{-4}M) potentiated the GABA responses in some cells, but only in 8 out of 19 tests did the potentiation exceed 10% (Figure V-3A). The remaining 11 tests showed little change. Potentiated responses were increased both in amplitude and duration. In two preparations the concentration was raised to 10^{-3} M: this caused a reduction in amplitude of the GABA response.

No concentration of pentobarbitone significantly affected the

Figure V-2

Effects of pentobarbitone on GABA and glycine responses.

Records from 2 cells showing:

(upper traces), GABA potentiation by 10^{-4}M pentobarbitone and antagonism by 10^{-3}M pentobarbitone (no intervening wash);

(lower traces), lack of effect of 2.5×10^{-4}M pentobarbitone on glycine responses.

Durations of exposures to pentobarbitone:

(upper traces) 13min (10^{-4}M), 6min (10^{-3}M);

(lower traces) 14min.

Preparations washed for 8min (upper traces), 14min (lower traces).

Iontophoretic pulses: GABA 0.5s, 60nA; glycine 3s 200nA.

Hyperpolarising current pulses: 1nA (upper traces), 0.7nA (lower traces).

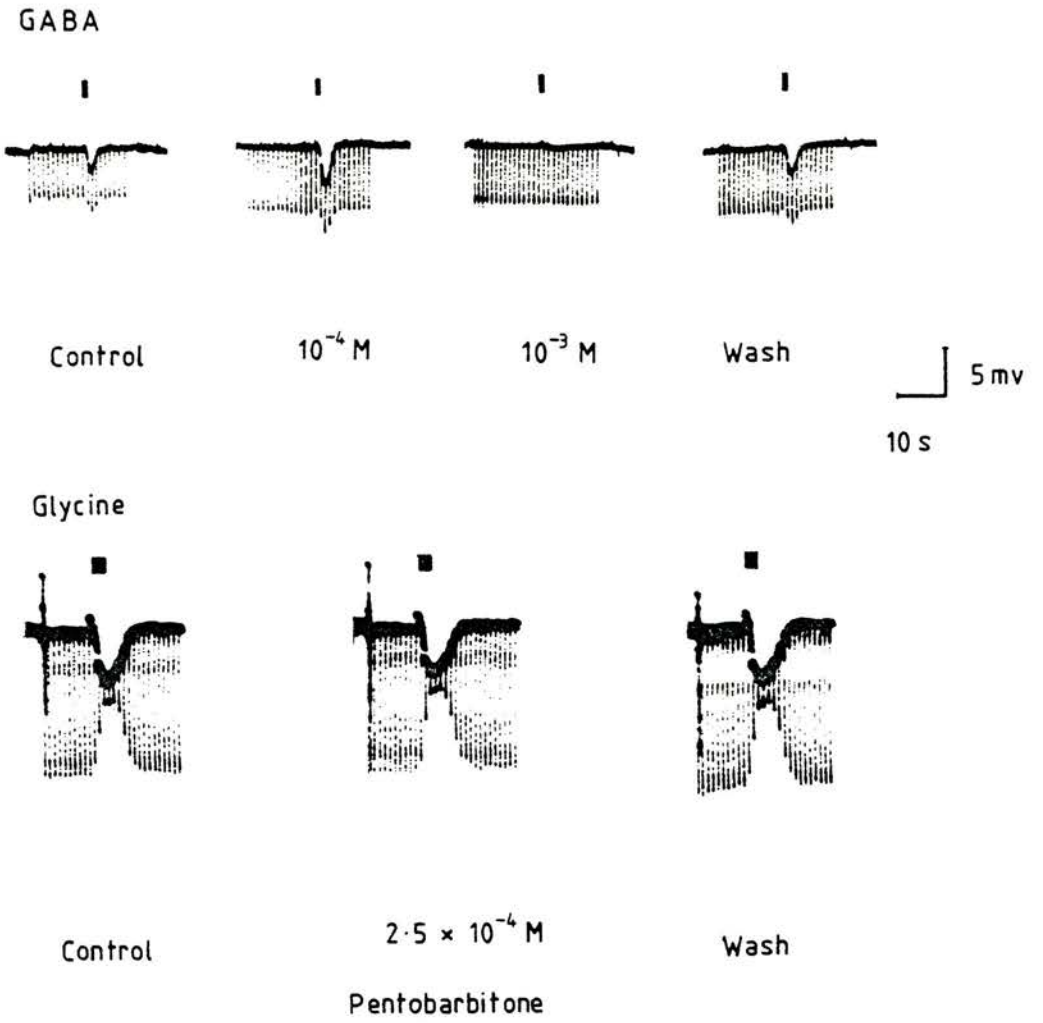
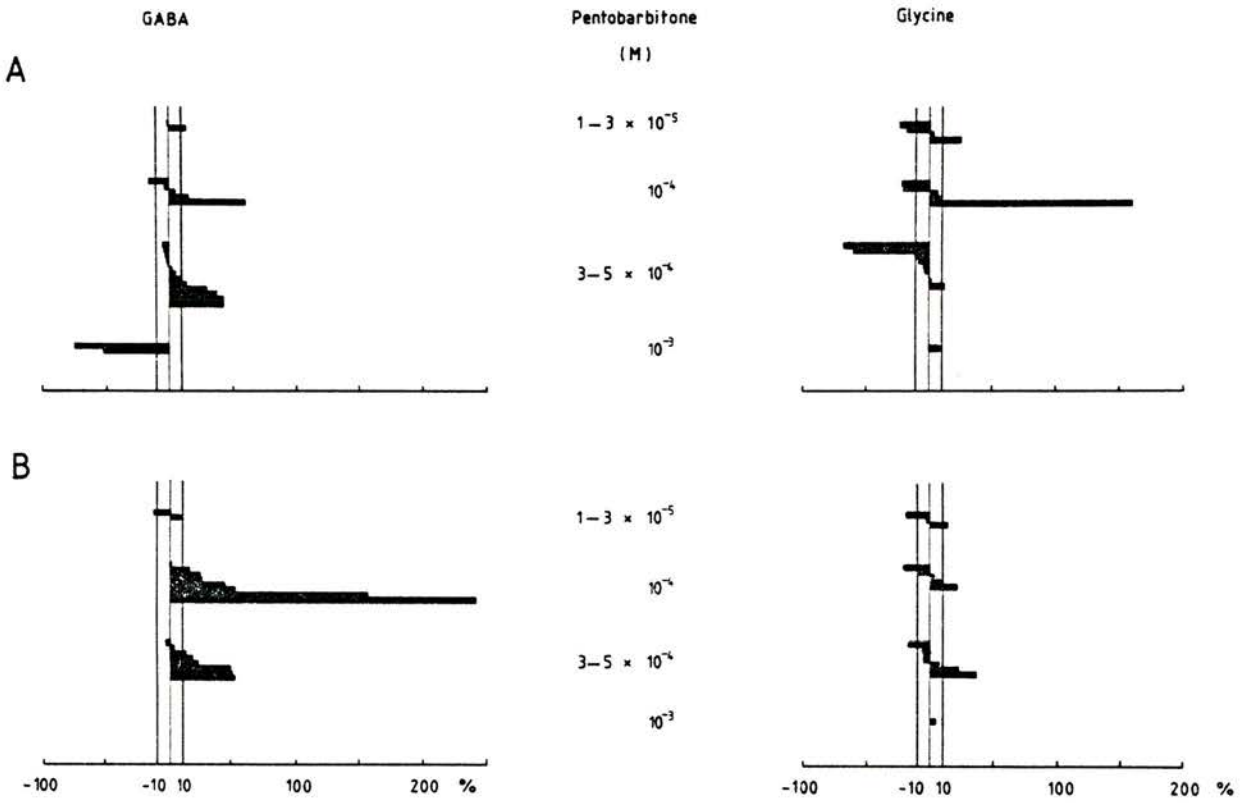

GABA
Control
10^{-4} M
10^{-3} M
Wash
5 mv
10 s
Glycine
Control
$2{\cdot}5 \times 10^{-4}$ M
Pentobarbitone
Wash

Figure V-3

Effects of pentobarbitone on responses of individual cells

A: peak change in input resistance, B: ratio of area to peak. Each bar represents the % potentiation of the control measurement for a single cell. Changes of less than 10% (bars lying wholly between the lines drawn at +10% and -10%) were not considered important. Note that GABA potentiation by 1-5 x 10^{-4} M pentobarbitone was not always detected, and that there was no consistent effect on the glycine responses.


GABA
Pentobarbitone
(M)
Glycine
A
1–3 × 10⁻⁵
10⁻⁴
3–5 × 10⁻⁴
10⁻³
B
1–3 × 10⁻⁵
10⁻⁴
3–5 × 10⁻⁴
10⁻³
-100
-10
10
100
200
%

glycine responses (Figure V-3A & B).

Ketamine

Ketamine selectively potentiated the GABA responses (Figure V-4), an effect consistently detectable at 3.7×10^{-5} M and reaching statistical significance at 3.7×10^{-4} M ketamine (Figure V-5B). Unlike the potentiation by pentobarbitone, that due to ketamine was almost entirely attributable to prolongation of the response, as is shown by the area:peak ratios. Slowing of the onset and offset of the GABA response was most striking at high concentrations of ketamine (3.7×10^{-3} M).

In contrast, Figure V-5B shows a dose-dependent antagonistic action of ketamine on glycine responses. This effect was statistically significant at 3.7×10^{-4} M when the response was measured as the peak change in resistance. Only two cells were tested with ketamine at 3.7×10^{-3} M but in both cases depression of the response was dramatic. No effect of ketamine on the response profile was detected for glycine (e.g Figure V-4).

Alphaxalone

The action of alphaxalone ($>10^{-5}$ M) was consistently to depress responses to both GABA and glycine (Figures V-6 & V-7). The commercial vehicle, which contains poly-oxyethylated castor oil, had an antagonistic action in some experiments (7 out of 10 tests using GABA and 3 out of 6 with glycine showed a reduction in response amplitude which exceeded 10%). This latter effect was small, and was statistically significant only in the case of resistance measurements on GABA responses.

Figure V-4

Effects of ketamine on GABA and glycine responses.

Records from 1 cell showing GABA potentiation by 3.7×10^{-4} M ketamine and complex effect of 3.7×10^{-3} M (no intervening wash). In this cell glycine responses were unaffected by 3.7×10^{-4} M ketamine but reversibly depressed by 3.7×10^{-3} M.

Duration of exposures to ketamine: 8min (3.7×10^{-4} M),
10min (3.7×10^{-3} M).

Preparation washed for 10min (upper trace), 11min (lower trace).

Iontophoretic pulses: GABA & glycine 3s, 60nA.

Hyperpolarising current pulses: 1nA.

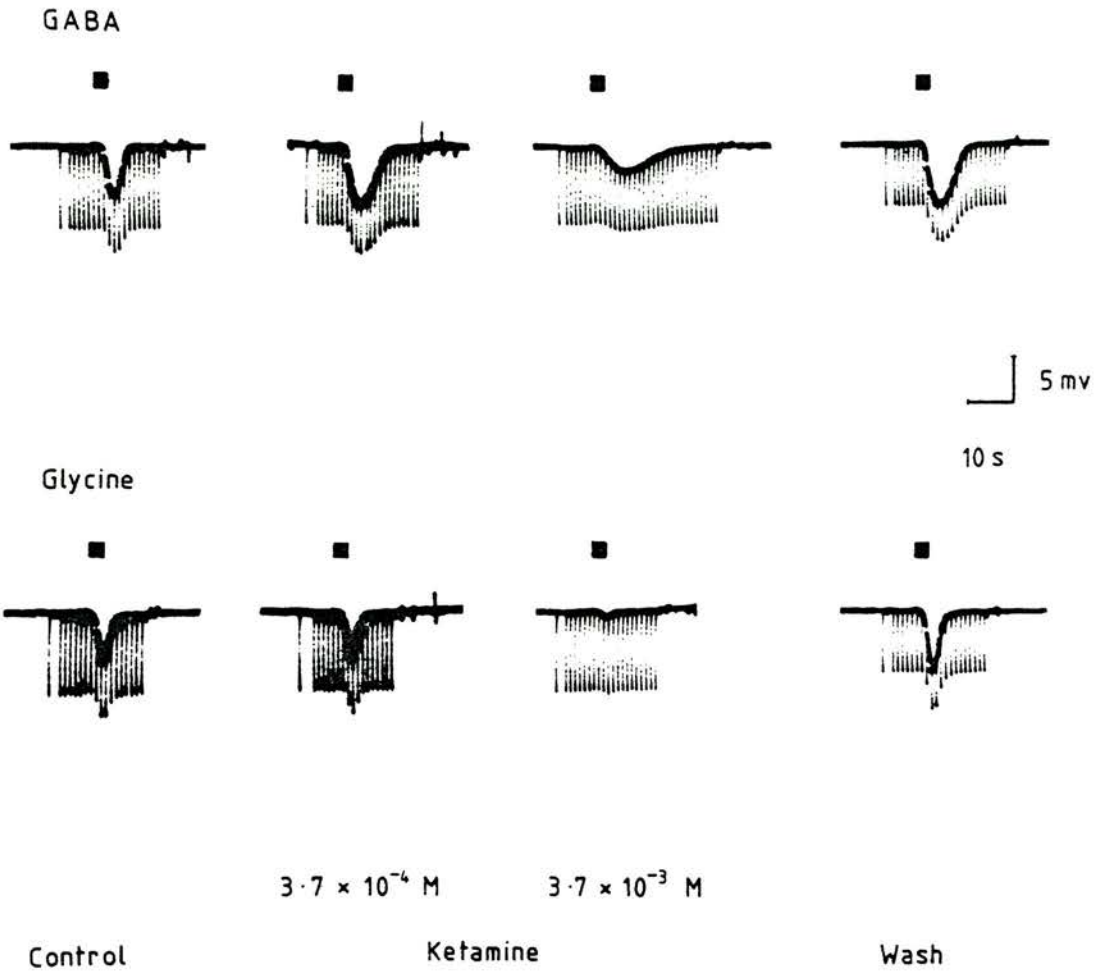

GABA
5 mv
10 s
Glycine
3·7 × 10⁻⁴ M
3·7 × 10⁻³ M
Control
Ketamine
Wash

Figure V-5

Effects of ketamine on responses of individual cells

A: peak change in input resistance, B: ratio of area to peak. Each bar represents the % potentiation of the control measurement for a single cell. Changes of less than 10% were not considered important. Note that prolongation of the GABA reponse (B) was a consistent finding during exposure to ketamine $>10^{-5}$ M whereas depression of glycine responses (A) is first consistently observed at 3.7×10^{-4} M.

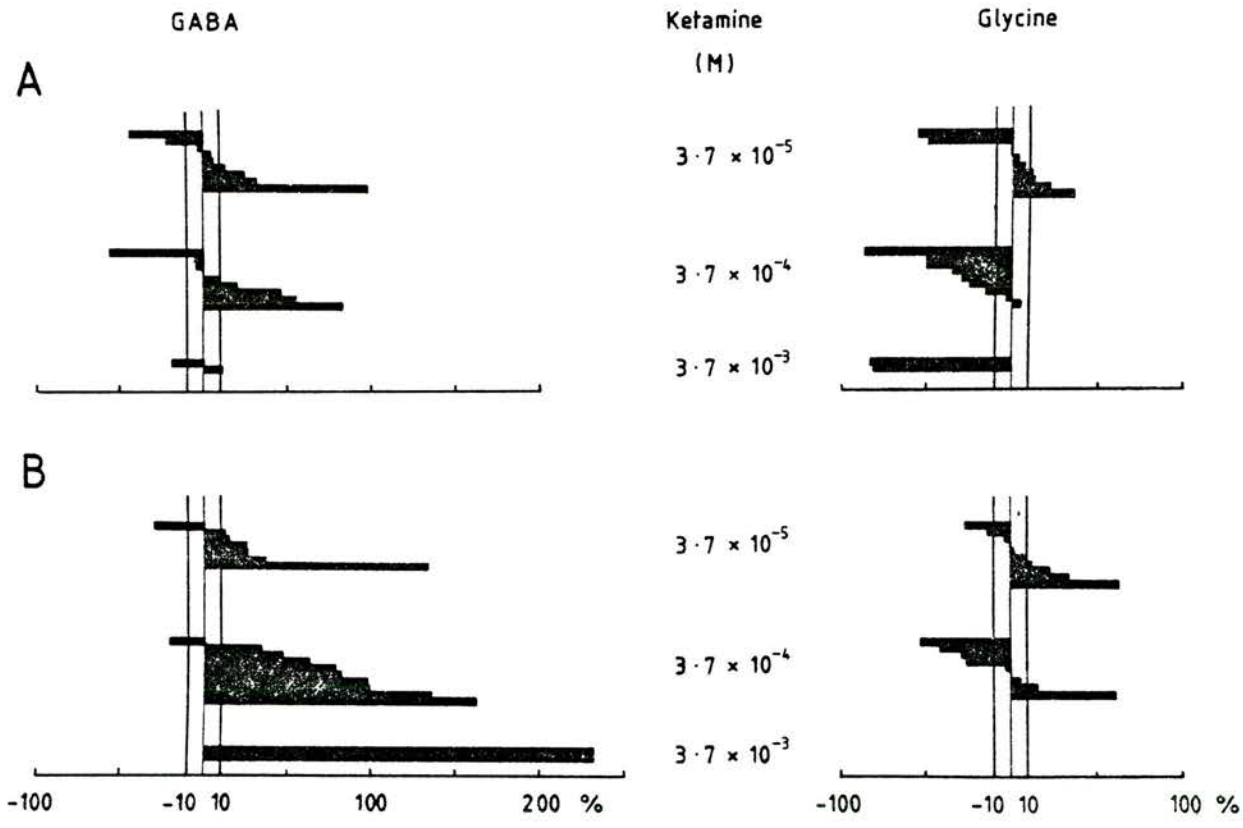

GABA
Ketamine
(M)
Glycine
A
3·7 × 10⁻⁵
3·7 × 10⁻⁴
3·7 × 10⁻³
B
3·7 × 10⁻⁵
3·7 × 10⁻⁴
3·7 × 10⁻³
-100
-10 10
100
200 %
-100
-10 10
100 %

Figure V-6

Effect of alphaxalone on GABA and glycine responses.

Records from 2 cells showing reversible depression of GABA (9min exposure to 2.7×10^{-5} M alphaxalone) and of glycine (8min exposure to 1.4×10^{-5} M) responses. Preparations washed for 22min (upper traces), 8min (lower traces).

Iontophoretic pulses: GABA 2s, 30nA; glycine 5s, 500nA.

Hyperpolarising current pulses: 1nA (upper traces), 0.9nA (lower traces).

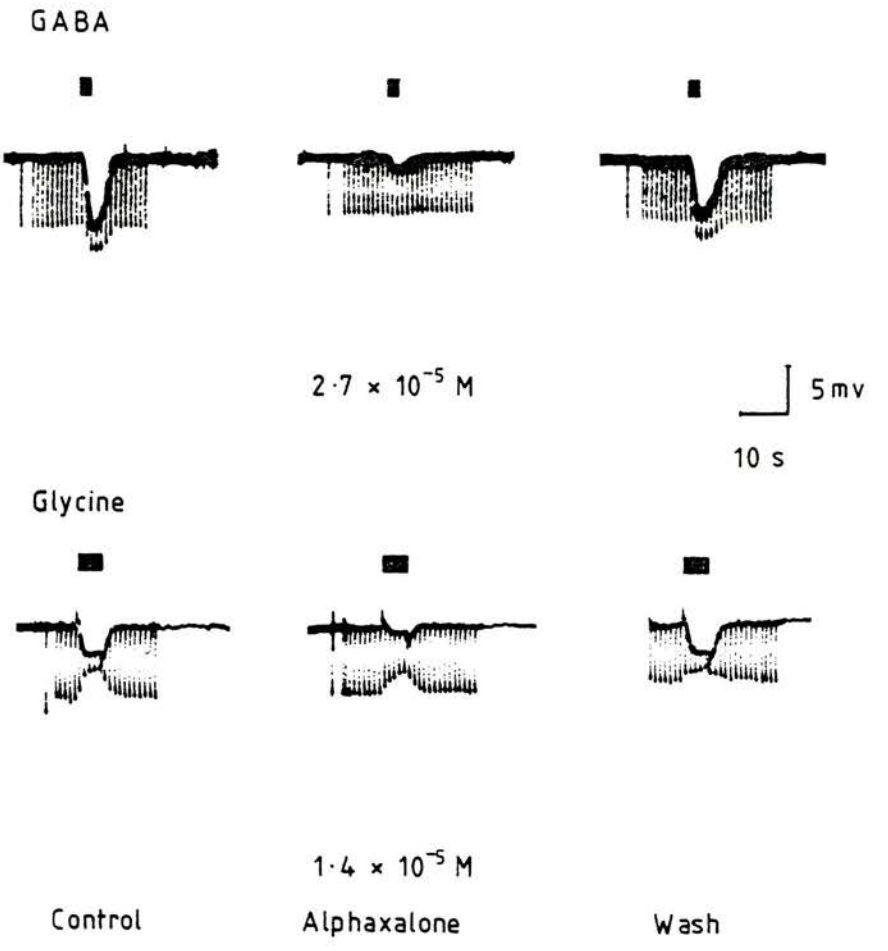
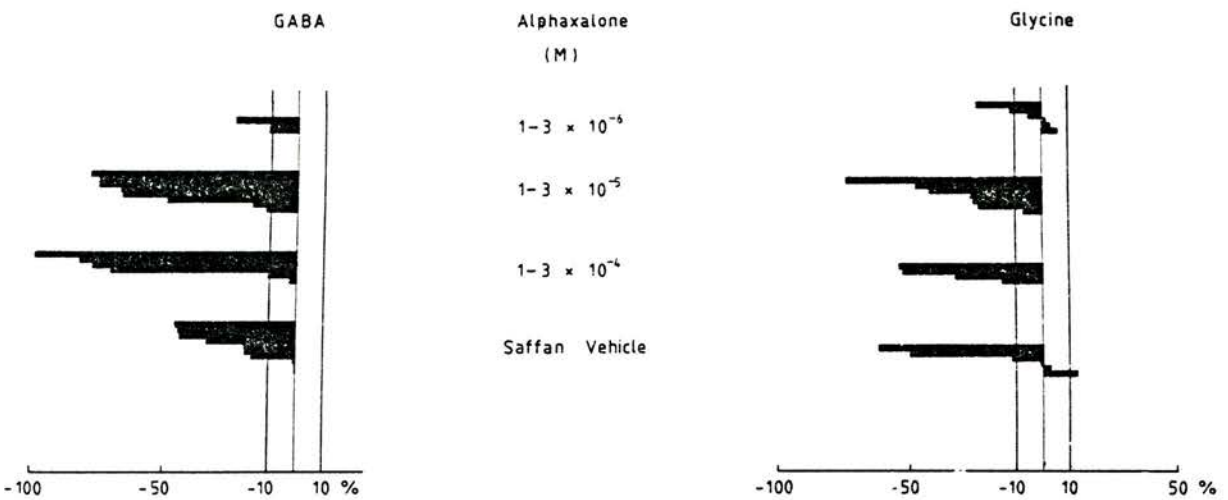

GABA
2·7 × 10⁻⁵ M
5 mv
10 s
Glycine
1·4 × 10⁻⁵ M
Control
Alphaxalone
Wash

Figure V-7

Effects of alphaxalone on responses of individual cells

Peak change in input resistance measured. Each bar represents the % potentiation of the control measurement for a single cell. Changes of less than 10% were not considered important. Depression of both GABA and glycine responses was a consistent finding during exposure to alphaxalone $>10^{-5}$ M. Depression by the commercial vehicle was less consistent and less extensive.


GABA
Alphaxalone
(M)
Glycine
1-3 × 10⁻⁶
1-3 × 10⁻⁵
1-3 × 10⁻⁴
Saffan Vehicle
-100
-50
-10
10 %
-100
-50
-10
10
50 %

Metomidate

Metomidate, too, had a dramatic depressant action on both GABA and glycine responses (Figure V-8), but only at high concentration (1.8×10^{-3} M). There was no evidence either of potentiation at any concentration (Figure V-9) or of alteration in the response profile.

The effect of agonist dose on the modulating influence of anaesthetics

It was recognised that the agonist concentration at the receptors might affect the recorded influence of the anaesthetic drugs. Experiments were therefore performed in which different doses of a single transmitter were applied to a cell.

The same anaesthetic-transmitter combinations as previously described were tested in these experiments, each combination receiving 2-8 tests in between 2 and 4 preparations. Only the potentiation effects were consistently affected by transmitter dose, and pentobarbitone was more affected than was ketamine. Thus pentobarbitone consistently potentiated GABA responses only when short iontophoretic pulses were used. Measurements of peak change in input resistance obtained in one such experiment are shown in Figure V-10. In this particular experiment 10^{-4} M pentobarbitone approximately doubled the response to a 0.5s GABA pulse, whereas the response to a 2s pulse was increased by only about a quarter. Higher concentrations of pentobarbitone caused a reversible loss of this effect.

Figure V-8

Effect of metomidate on GABA and glycine responses.

Records from 1 cell showing lack of effect of 1.8×10^{-5} M metomidate on GABA and glycine responses. Antagonism by 1.8×10^{-3} M metomidate was total in both cases. Durations of exposures to metomidate (no intervening wash): 10min (1.8×10^{-5} M), 5min (1.8×10^{-3} M).

Preparation washed for 18min (upper traces), 17min (lower traces).

Iontophoretic pulses: (GABA & glycine) 2s, 300nA

Hyperpolarising current pulses: 1nA

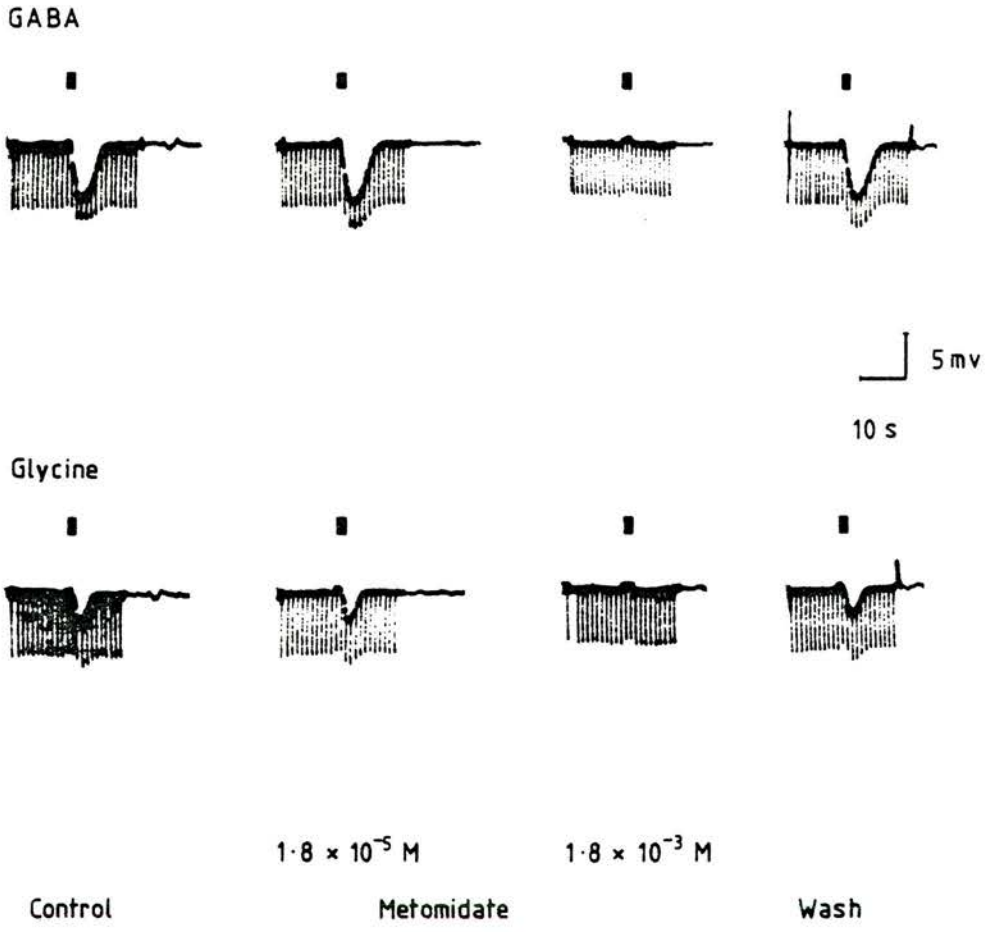

GABA
5 mv
10 s
Glycine
1·8 × 10⁻⁵ M
1·8 × 10⁻³ M
Control
Metomidate
Wash

Figure V-9

Effects of metomidate on responses of individual cells

Peak changes in input resistance measured. Each bar (of unit width) represents the % potentiation of the control measurement for a single cell. Changes of less than 10% were not considered important. Depression of GABA and glycine responses was consistent only in the presence of 1.8×10^{-3} M metomidate, although GABA responses were sometimes markedly depressed by 1.8×10^{-4} M. Note the absence of any potentiation.

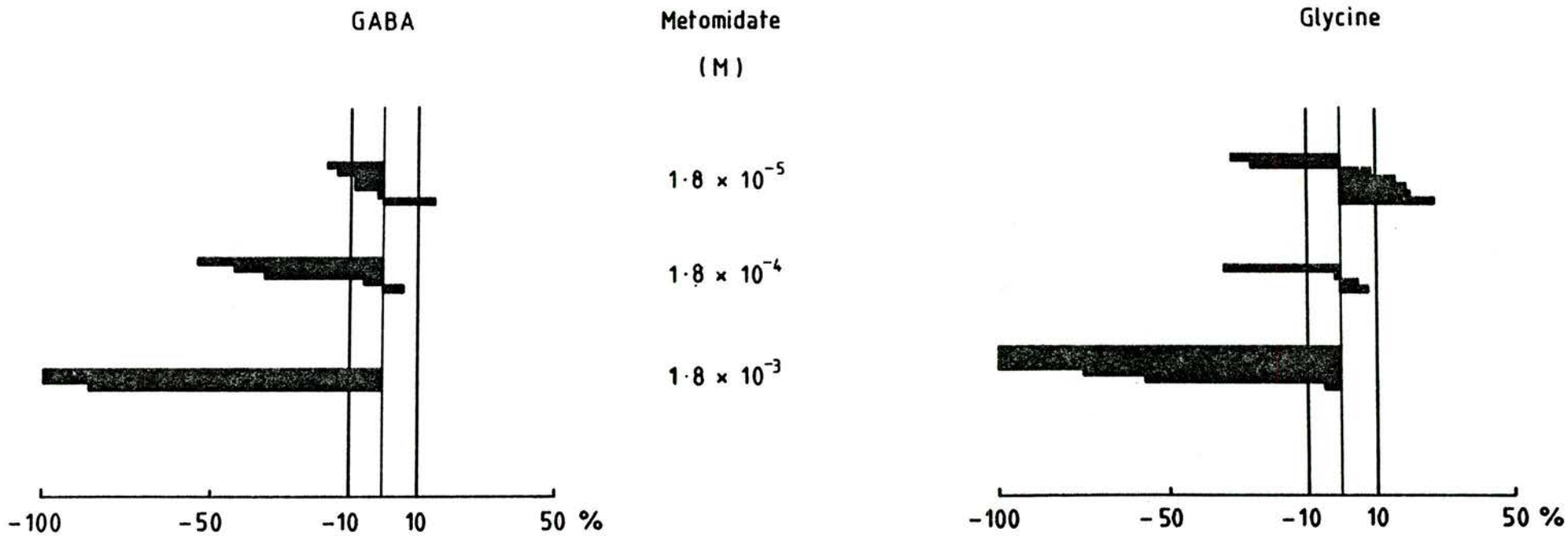

GABA
Metomidate
(M)
Glycine
1·8 × 10⁻⁵
1·8 × 10⁻⁴
1·8 × 10⁻³
-100
-50
-10
10
50 %
-100
-50
-10
10
50 %

Figure V-10

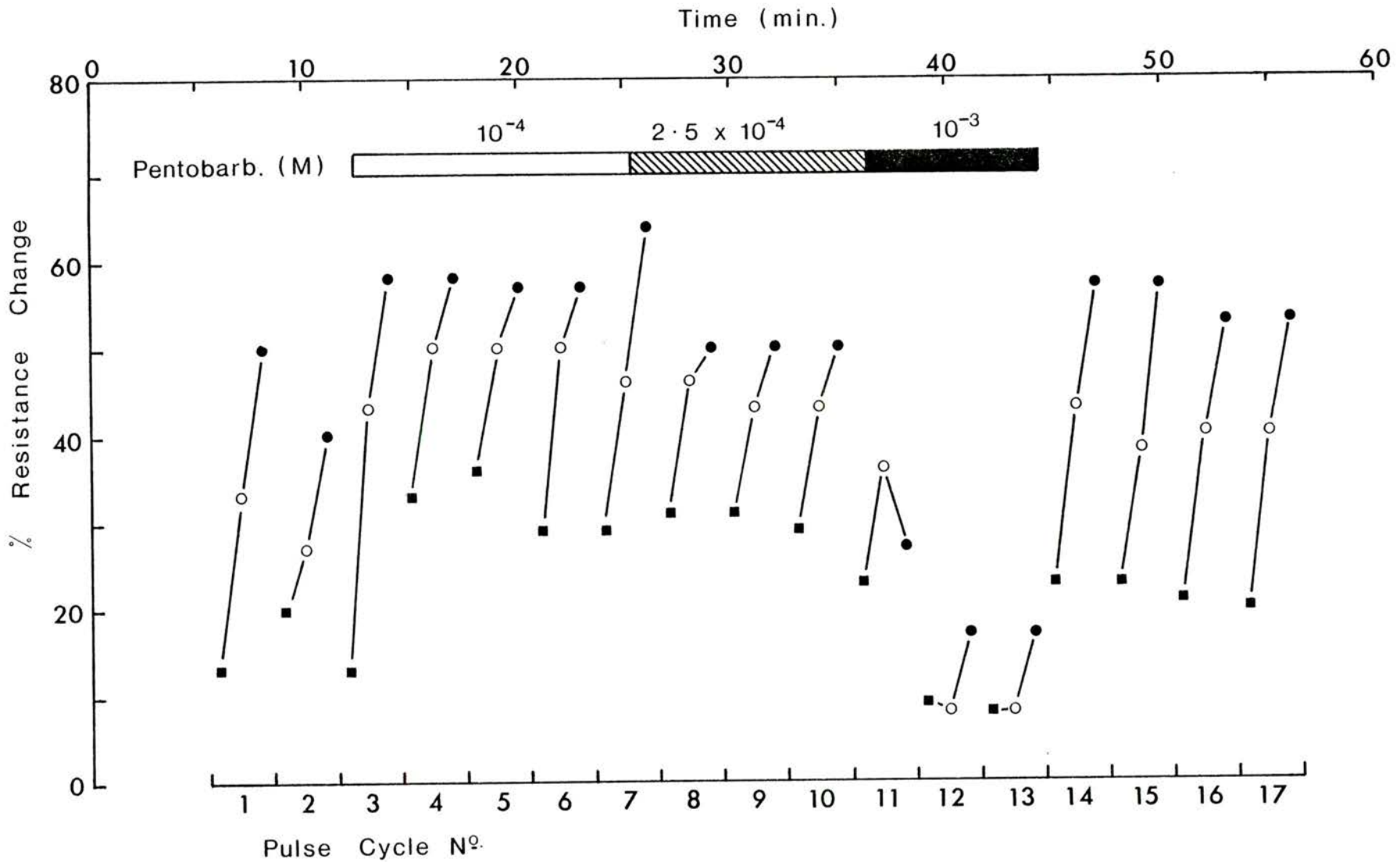
GABA-induced resistance changes measured in a single cell before, during and after application of increasing concentrations of pentobarbitone.

Ordinate: GABA-induced resistance change (%);

Abscissa: time after pulse sequence started (min).

GABA pulses (amplitude 60nA) applied for 0.5s (■), 1.0s (○) and 2.0s (●) in repeated sequence.

Pentobarbitone at 10^{-4}M approximately doubled the response to a 0.5s GABA pulse whereas the response to a 2.0s pulse increased by only about a quarter. Higher concentrations of pentobarbitone reversibly eliminated the potentiation.


Time (min.)
0
10
20
30
40
50
60
Pentobarb. (M)
10^{-4}
$2 \cdot 5 \times 10^{-4}$
10^{-3}
% Resistance Change
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Pulse Cycle Nº.

Discussion

The effect of agonist dose on the recorded responses

Whereas the response to a small dose of GABA or glycine was unambiguous, the complex responses observed when large doses of either compound were applied resembled responses recorded by Barker & Ransom from cultured spinal cells (Barker & Ransom, 1978a: Figure 4). These authors suggested that the complexities could be explained by changes in the driving force on the permeant ion species (probably chloride) brought about by changes in the transmembrane electro-chemical gradient.

More recently, Alger & Nicoll (1982) have examined a similar phenomenon, in the pyramidal cells of the rat hippocampus, and have presented several lines of evidence suggesting that there are at least two types of GABA receptor, mediating respectively hyperpolarising and depolarising responses. They equated the hyperpolarising responses with those of extrasynaptic receptors found principally on the soma, whereas the depolarising responses were thought to be the result of GABA's binding to synaptic receptors on the dendrites. The question as to whether hyperpolarising-type receptors were also present on the dendrites was not resolved. The two receptor types could be distinguished pharmacologically although both were blocked by the GABA antagonist bicuculline. Thus the GABA analogue THIP (4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridine-3-ol) was more potent in eliciting hyperpolarising responses; and pentobarbitone and diazepam preferentially increased the depolarising and hyperpolarising responses respectively.

Figure V-11

Conversion of glycine responses from depolarising to hyperpolarising after movement of iontophoresis electrode.

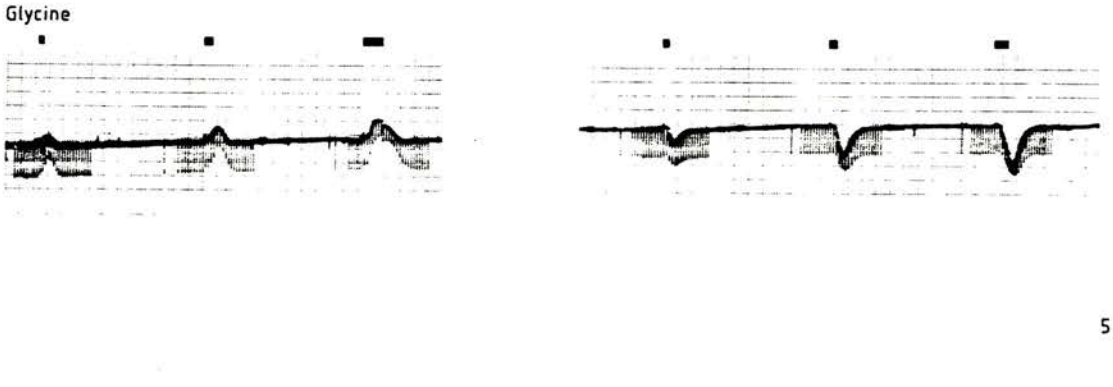
Pulse lengths: (depolarising; left to right) 2s, 3s, 7s;

(hyperpolarising; left to right) 2s, 3s, 5s.

Pulse amplitudes: 200nA

Hyperpolarising pulses: 1nA

3 minutes elapsed between the two sections of trace.


Glycine
5 mV
10s

Alger & Nicoll (1982) reported only on GABA receptors. The results presented here suggest that glycine, also, may act on more than one receptor type. Indeed in one cell a slight adjustment of the electrode position converted a pure depolarising response into a hyperpolarising one (Figure V-11). Since there was no equivalent change in membrane potential this was evidence for there being glycine responses with two reversal potentials. However, the tip of the iontophoresis electrode could not be clearly seen, so its position in relation to the cell's dendritic tree could not be estimated.

Alger & Nicoll reported that the depolarising component of the IPSP was more prominent at low temperature and that it showed strong temperature dependence. In this connection it is interesting that the present lamprey experiments were conducted at temperatures which were low, although not unphysiological for the species. However, there was no consistent difference between the shapes of responses recorded at 2°, 10°C and at room temperature.

Anaesthetics differ in their post-synaptic effects

These experiments were designed to compare the abilities of the four anaesthetic drugs to interfere with the responses of bulbar Müller cells to GABA and glycine. Other comparative studies have not utilised all of these particular drugs and the lamprey preparation has not previously been used for this type of investigation.

Many workers have stressed the GABA potentiating properties of the barbiturates in a range of preparations (e.g. Barker & Ransom, 1978b). In this study, as in that of Bowery & Dray (1978), the GABA-

potentiating action of pentobarbitone was evident only in a proportion of cells, and showed marked dependence on the agonist dose, being detectable only if low amplitude or short iontophoretic currents were employed. The foregoing discussion may cast some light on the capricious nature of this effect, but large doses of GABA were required to elicit responses with an appreciable depolarising component whereas pentobarbitone's potentiating effect was much more marked when the GABA doses were small (Figure V-10).

Ketamine, also, selectively potentiated the responses of Müller cells to GABA, but the effect differed from that of pentobarbitone in that it was principally on the time course of the response; both the onset and offset of the response were slowed by ketamine ($>10^{-5}$ M). Other studies (e.g. Jessel & Richards, 1977; Minchin, 1981) suggest that this potentiation is not the result of altered GABA uptake mechanisms. Potentiation of GABA responses in the superior cervical ganglion has recently been demonstrated to follow administration of ketamine in concentrations similar to those use in this study (Little, 1982). Only peak amplitude measurements were quoted, but the potentiation, occurring over the range 1.8×10^{-5} M to 3.7×10^{-3} M was maximal at 1.8×10^{-4} M, a result consistent with the present findings. These results are also consistent with the reported inefficacy of ketamine against strychnine convulsions.

In contrast, neither alphaxalone nor metomidate caused significant potentiation of GABA responses at any concentration tested; instead, both drugs produced parallel depressions of the responses to GABA and glycine. There are no previous reports of the effects of either anaesthetic on responses to the inhibitory

transmitters; but etomidate, an imidazole derivative closely related to metomidate, has been studied: it is reported to be similar in action to pentobarbitone, selectively potentiating GABA responses at low doses (10^{-5}M) but having a depressant effect at high doses (10^{-3}M). As with the barbiturates, the potentiating action shows stereo-specificity (Hill & Taberner, 1975; Huang & Barker, 1980). The optical isomers of metomidate have not been tested separately, but those of etomidate have been shown to differ in their pharmacological actions (Hill & Taberner, 1975).

There is little published information about the actions of alphaxalone/alphadolone on inhibitory mechanisms but Scholfield (1980) has described a potentiation of recurrent inhibition in the olfactory cortex caused by a range of anaesthetics including alphaxalone, pentobarbitone and ketamine. This study will be discussed in some detail in Section VII. Smaje (1976) and Richards & Smaje (1976) reported depressed responses to acetyl choline and glutamate in the olfactory cortex; and Pennefather & Quastel (1980) described alterations in endplate currents recorded at the neuro-muscular junction.

Relevance to clinical anaesthesia

Although the reticulo-spinal cells are not concerned with consciousness, the effects just described are likely to operate during anaesthesia and may be related to side effects produced by the drugs. The results are hard to reconcile with a unitary biophysical hypothesis of anaesthetic action. Such an hypothesis predicts similarity of action of different anaesthetic compounds (Metcalfe, Hoult & Colley, 1974), however exerted, and this was not

a present finding. The concentrations at which the anaesthetics have been tested in this study include those likely to obtain in the brain during clinical anaesthesia (Table III-5), and were themselves capable of inducing anaesthesia in lamprey ammocoetes (Table III-2).

The finding that neither GABA nor glycine responses were potentiated by alphaxalone and metomidate lends no support to the unitarian contention that general anaesthesia is the result of the enhancement of central inhibitory mechanisms (e.g. Cheng & Brunner, 1975; Scholfield, 1980). Scholfield recorded post-synaptic potentials in cortical neurones and found marked potentiation of those features generally held to be due to activation of an inhibitory interneurone during exposure to anaesthetics including pentobarbitone, alphaxalone and ketamine. However, the effect was also observed when the concentrations used were sub-anaesthetic. Scholfield's study will be discussed in detail in Section VII. The available evidence suggests that GABA and glycine receptors on the bulbar Müller cells of the lamprey are pharmacologically similar to those in higher vertebrates (Martin, 1979a; Nistri & Constanti, 1979).

Only with alphaxalone was depression of GABA and glycine responses in the lamprey seen at concentrations likely to be encountered during clinical anaesthesia in fish (Table III-2) and higher vertebrates. Should a comparable depression of inhibitory mechanisms underlie the excitatory phenomena commonly observed during anaesthesia with alphaxalone/alphadolone in mammals, a logical preventive treatment would be premedication with a GABA-potentiating agent such as a benzodiazepine (Curtis et al, 1976).

It thus is interesting that there are clinical reports of a reduced incidence of tremors and non-purposive movements when diazepam is used as a pre-medicant before anaesthesia with etomidate (Holdcroft et al, 1976) or alphaxalone/alphadolone (Vickers, Wood-Smith & Stewart, 1981).

SECTION VI

Anaesthetics and Excitatory Transmitters

Introduction

Since Hayashi (1952) first discovered the clonic convulsions produced by application of L-Glutamate to the cortex, the amino acids L-glutamate and L-aspartate, ubiquitous within the vertebrate brain, have been found to excite virtually all neurones to which they have been applied. A neurotransmitter role for glutamate has been proposed (see Curtis & Crawford, 1969), and the extensive literature concerning the different types of membrane receptor for glutamate has been reviewed recently by Nistri & Constanti (1979), Di Chiara & Gessa (1981) and Watkins & Evans (1981).

Pharmacological studies have distinguished only three types of glutamate receptor in the vertebrate CNS as compared with more than six in invertebrates. The receptors are thought to coexist on cells and, whilst their selectivities are not absolute, the receptors are usually categorised according to the preferred agonist:

(i) N-methyl-D-aspartate (NMDA),

(ii) quisqualate,

(iii) kainate.

These are agonists of relatively rigid 3-dimensional configuration and have been used as pharmacological tools although they are not suspected of having a transmitter function. The putative natural ligands L-glutamate and L-aspartate are more flexible and are able to bind to more than one receptor type. There is however some selectivity: quisqualate and kainate receptors preferentially bind glutamate whereas NMDA receptors prefer aspartate. Apart from showing different selectivities for agonists (NMDA receptors also bind D,L-homocysteate (DLH), and kainate receptors bind ibotenate)

the receptors also discriminate among antagonists. As with the agonists this discrimination is not absolute and, for example, D-α-amino adipate (DαAA) selectively antagonises NMDA responses whereas glutamate diethylester (GDEE) blocks the effects of quisqualate. The responses are also differentially affected by divalent cations and anaesthetics. Thus Anis, Burton & Lodge (1982) have recently reported a selective antagonistic action of ketamine on NMDA responses.

The excitatory transmitter roles of glutamate and aspartate in specific nervous pathways have not yet been clearly established because the amino acids also have metabolic functions and are very widely distributed, as are mechanisms for their uptake and synthesis. Probably the best current marker for glutamate pathways is the demonstrable presence of calcium-stimulated glutamate release (Cotman, Foster & Lanthorn, 1981). Glutamate responses are not invariably depolarising in the vertebrate CNS: hyperpolarisation has been recorded in the cerebellum, and a long-lasting inhibitory effect of glutamate has also been found in some cells, mediated by dendritic receptors and selectively activated by ibotenate (Nistri & Constanti, 1979).

Both glutamate and aspartate have been shown to excite the bulbar Muller cells of the lamprey (Wickelgren, 1977), causing a small decrease in input resistance associated with a membrane depolarisation. Bursts of synaptic potentials were superimposed on the depolarisation, except when the Ringer contained 20mM Mg^{2+}. A more detailed analysis of these responses (Matthews & Wickelgren, 1979b) showed that glutamate was a good candidate for the role of

the natural excitatory transmitter: both glutamate responses and the natural EPSPs had reversal potentials that were of the same order (about -35mV) and that were varied in a similar manner by cell damage. The latter, by permitting leakage of cell constituents, reduced the trans-membrane ion gradients. EPSPs were insensitive to variations in the chloride gradient, and it was thought that they were mediated by an increase in membrane permeability to both sodium and potassium ions, although this was not proved. By comparison with the GABA responses (Section V) the Müller cell response to glutamate was characterised by its faster onset and smaller associated decrease in membrane resistance.

Responses could be recorded whether glutamate was applied to the soma or the dendrites, but iontophoretic application to the giant axon, an area devoid of excitatory inputs, was without effect. However, Barker & Ransom (1978a) observed a non-uniform distribution of responses when glutamate was applied over the surface of cultured mouse neurones; and Zieglgänsberger & Champagnat (1979), applying glutamate to the somata or dendrites of spinal neurones in cats, also observed differences in effect. Whereas both sites gave depolarising responses, only after application of a high concentration to the soma was there a measurable and progressive rise in membrane conductance. The question has yet to be resolved whether a conductance decrease occurring in the dendrites is undetectable purely because of electrotonic decrement; decrement has previously been invoked to explain differences observed between the reversal potentials of the EPSPs and the glutamate responses (e.g. Curtis et al, 1968). Wickelgren (1977) suggested that the

dendritic response in lampreys was the result of electrotonic spread from neighbouring cells that were also affected by glutamate. However, the spread of iontophoretically applied glutamate is limited.

The possibility of an interaction between anaesthetic drugs and neuronal responses to transmitters was recognised early in the study of CNS transmitters, as was its importance: many studies that involved measurement of neuronal firing rates during the iontophoretic application of transmitters were conducted on anaesthetised animals. Of the anaesthetics used in this study, the effects of pentobarbitone are particularly well documented, this being an anaesthetic commonly used in such experiments. Although some of the evidence was conflicting, the consensus was that pentobarbitone caused a greater depression of ACh than of glutamate or DLH responses; indeed glutamate and DLH were sometimes employed to "activate" neurones that were otherwise silent, so that the effects of inhibitory transmitters could be investigated. Several unrelated anaesthetics have been shown to depress responses to glutamate, for example pentobarbitone and alphaxalone do so in the prepyriform cortex, but this is not a property shared by all anaesthetics. Halothane at concentrations associated with light anaesthesia (up to approx. 1%) was one exception (Richards & Smaje, 1976).

ACh, also, is an excitatory transmitter in parts of the vertebrate CNS, and there have been many reports of anaesthetic reduction of its effect within the brain (e.g. Exley, 1954; Krnjević & Phillis, 1963; Curtis & Ryall, 1966; McCance et al, 1968). Yet Smaje (1976) found that whereas alphaxalone reduced the

sensitivities of cells in the prepyriform cortex to ACh, pentobarbitone at anaesthetic concentrations (5×10^{-5} M to 4×10^{-4} M) had no similar consistent effect. Curtis & Crawford (1969), reviewing the available evidence, had earlier drawn attention to the variable extents of anaesthetic depression occurring in different regions of the brain: for example the brainstem (Salmoiraghi & Steiner, 1963) and spinal cord (Curtis & Ryall, 1966) were minimally affected.

The Müller cells of the lamprey have been found not to respond to ACh (Matthews & Wickelgren, 1979b) but there is evidence suggesting that ACh does have a functional role elsewhere in the lamprey CNS. Wachtler (1974) detected acetylcholinesterase in many parts of the brainstem but not in the region of the Müller cells.

This, then, is the context in which the present experiments were performed, in which glutamate was applied to the bulbar Müller cells in the absence and presence of the anaesthetics. ACh has not been tested. Because it is likely that other excitatory transmitters also affect these cells, a few experiments have been conducted using substance P, an undecapeptide whose existence was first recognised in 1931 (von Euler & Gaddum, 1931) and which has been shown more recently to be neuro-active (e.g. Konishi & Otsuka, 1974; Nicoll, Alger & Jahr, 1980). Full examination of the actions of substance P in the lamprey preparation has been deferred.

The experiments using iontophoresis were carried out concurrently with those of Section V, with solution D (15mM Mg^{2+}) used as the perfusate. Other studies, using isolated spinal cord preparations, have shown that the NMDA-preferring receptor is

selectively blocked by even a low concentration (2mM) of magnesium, and also by other divalent cations including calcium (Evans & Watkins, 1978; Ault et al, 1980). This suggests that the responses measured were probably those of receptors preferring kainate or quisqualate.

Methods

The procedures for dissection, recording intracellular potentials, iontophoresis of glutamate and application of anaesthetics have been described in Section II. Frequently GABA, glycine and glutamate were tested on the same cell, standard pulses of each transmitter being applied in repeated sequence. In case there was undetected contamination of the responses, caused either by mixing of solutions within the 3-barrelled electrode or by leakage of transmitters from the electrode tips, some experiments were carried out in which the iontophoresis electrode contained glutamate alone. As in Section V, two types of experiment were performed:

i) fixed test pulses repeated at intervals of 1 or 3 minutes,

ii) a repeated sequence of test pulses in order of increasing length - in case the anaesthetic effect was related to the dose of transmitter.

Current controls were provided by incorporation of 0.5M NaCl in one barrel (and in the pulse sequence) or by application of pulses of reversed polarity to the glutamate-containing barrel. Neither with glutamate nor with GABA or glycine, did the latter procedure cause detectable ejection of transmitter from other barrels in the electrode assembly. Most experiments were performed with the

preparation perfused with Solution D (15mM Mg^{2+}), although the effect of glutamate at normal [Mg^{2+}] was also tested.

Analysis of the results was less detailed than with the inhibitory transmitters. In most cells the change in input resistance could not be measured with any degree of accuracy, so measurements were confined to the changes in membrane potential and in the area enclosed by the envelope of the response. The latter has been described in Section II. As in Section V, the results of different experiments have been combined after the proportional change in glutamate response amplitude at the peak of the drug effect has been calculated.

Pilot studies with substance P were carried out with the peptide bath-applied. A solution of the peptide was prepared at 10^{-3} M and 0.1ml volumes injected into the inlet tube close to the chamber. The concentration of peptide at the cell surface therefore was unknown, and no attempt was made to quantify it. Similar injections were made when the preparation was perfused with high [Mg^{2+}] Ringer, and with normal Ringer (solution C).

Results

Responses of bulbar Muller cells to glutamate

Contrary to previous reports in which bath application of glutamate in solutions of "normal" magnesium concentration was said to reliably excite bulbar cells (Wickelgren, 1977), iontophoresis of glutamate in high magnesium Ringer elicited a response in only about 70% of cells. The form of the response varied greatly between cells but the direction was always depolarising. Figure VI-1 illustrates

two typical shapes: the one a rapid large depolarisation with equally rapid decay, and the other a much smaller sustained change in potential with sharp onset and offset and showing no sign of desensitisation. Responses were frequently intermediate in shape. The only consistent relation between the change in input resistance and the magnitude or speed of the depolarisation was that the "squat" responses (e.g. Figure VI-1A(i)), seen in 4 cells out of 45, were associated with a minimal change in input resistance. Records of resting potential were not kept but there was no obvious relation between the resting potential and the shape of the glutamate response: small amplitude responses were seen in cells with high resting potentials as well as in those that were relatively depolarised.

Because an unexpectedly high proportion of cells did not respond to glutamate, the different types of response were not distinguished when the effects of the anaesthetics were assessed. No differential effect was readily discernible and it has already been mentioned that, in motoneurones, the use of high [Mg^{2+}] Ringer operates to select the kainate or quisqualate-preferring receptors. The different shapes could be the result of glutamate's acting at receptors at different distances from the recording electrode, but the onset of the smaller responses was still rapid. These small responses could not be increased appreciably by prolonging or increasing the amplitude of the iontophoretic pulses, although the positioning of the iontophoresis electrode was much more critical for glutamate than for GABA or glycine responses.

Figure VI-1

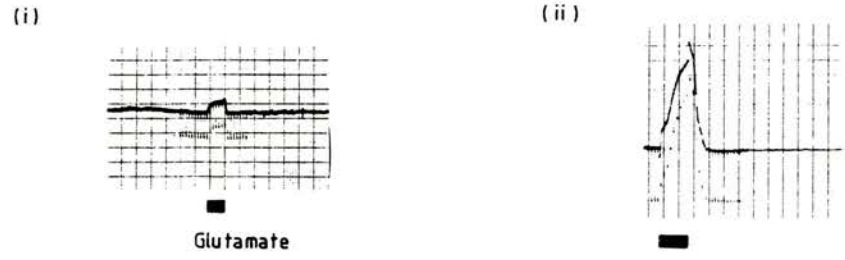
Responses of different cells to pulses of glutamate

A: (i) low amplitude sustained response to 70nA applied for 6s. The amplitude was not increased by increasing the pulse strength or duration. Change in input resistance undetectable.

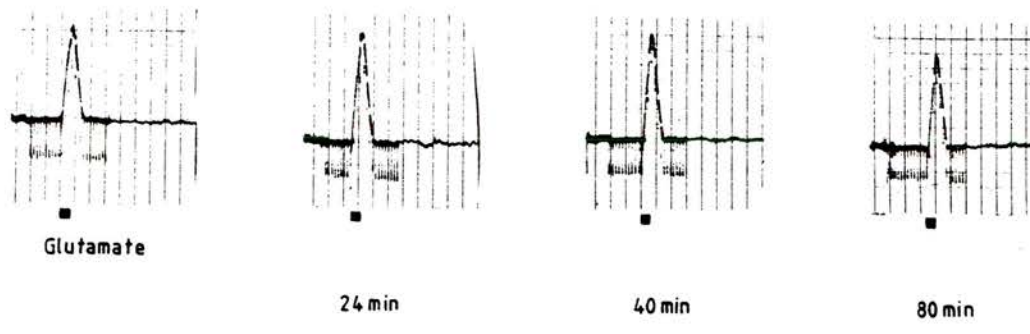
(ii) larger amplitude depolarisation associated with 39% decrease in input resistance in response to glutamate pulse (300nA, 10s).

B: stability of glutamate responses over a period of 80min. 350nA pulses applied for 3s (t=0,24min) and 2s (t=40,80min). Note the rapid onset of the responses. Changes in input resistance minimal.

A



B



The amplitude of the depolarisation was markedly affected by fluctuations in the resting membrane potential in some experiments; but in the absence of such fluctuations, and of anaesthetic effects, remained stable over periods of an hour or more (Figure VI-1B).

Elevation of the [Mg^{2+}] reduced the tendency for the glutamate-induced depolarisation to be interrupted by synaptic potentials and intermittent firing of action potentials (Figure VI-2). However, in some experiments such potentials occurred even though the [Mg^{2+}] was appeared to be adequate to block the spontaneous activity between applications of glutamate. Response amplitudes were not measured in these experiments because the full extent of the depolarisation was masked. There was no evidence that magnesium acted as a glutamate antagonist at the receptors responsible for the responses obtained, but it is possible that such antagonism accounted for the unexpectedly low proportion of cells in which responses were detected.

The effects of anaesthetics on the responses to glutamate

Responses were measured in cells from a total of 33 ammocoetes. Numbers exposed to the anaesthetics were: pentobarbitone (10 ammocoetes); ketamine (7); alphaxalone (10); metomidate (4). In addition two ammocoetes were exposed to the Saffan vehicle.

The most striking feature of these experiments was the lesser effect of anaesthetics on glutamate than on GABA and glycine responses, despite the fact that small changes in membrane conductance would exert a more readily detectable effect on the glutamate response, whose reversal potential is far from the resting potential. No drug caused potentiation of the glutamate responses.

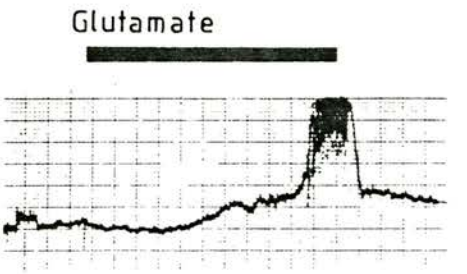
Figure VI-2

Effect of magnesium on responses to glutamate

A: Application of glutamate was followed after a 20s delay by an increase in synaptic activity and a sustained depolarisation. The latter, initially slowly developing, was sufficient to generate action potentials. These have been truncated by the pen recorder.

B: Responses of a different cell to 3 pulses of glutamate 10min after $[Mg^{2+}]$ raised to 15mM. Synaptic potentials recorded during first two responses, only, and no action potentials were generated. Note the hyperpolarising after potential following the second response; this was not a consistent feature of the response. Artefact at onset/offset of iontophoretic current: 6mV

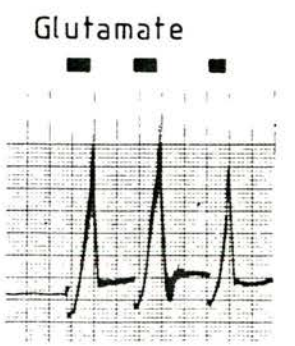
A



5mV

10s

B



10 mV

40s

Sample records are displayed in Figures VI-4 & 6, and the collected results for each drug in Figures VI-5 & 7. The numerical data from which the latter were prepared are presented in Appendix II.

Pentobarbitone

A clear dose-related depression of the glutamate response was observed, and only in 3 out of 16 tests in which measurements were made did depression not exceed 10% (Figure VI-5). In one of the three tests, one where 10^{-4}M pentobarbitone was used, there was an increase in the glutamate response. Serial measurements made during a single experiment are displayed in Figure VI-3, and illustrate the reversible depression of glutamate responses during exposure to a relatively high concentration of pentobarbitone (5×10^{-4}M). This depression was the more striking since this cell was hyperpolarised by pentobarbitone, a change that might be expected to accentuate the glutamate response. A small reduction in input resistance caused by pentobarbitone would have accounted for the result, but was not detected. Such a clear change in resting potential was rarely observed. Sample records from another cell exposed to 2.5×10^{-4}M pentobarbitone are shown in Figure VI-4A.

Ketamine

Sample records from a single experiment are displayed in Figure VI-4B, and the accumulated measurements are summarised in Figure VI-5. The latter suggests a dose-related depressant effect detectable at 3.7×10^{-5}M (the clinically relevant dose); but the one anomalous cell and the variable extent of the effect in different cells combined to render the effect statistically non-significant (Student's t-test, see Appendix II). The depressant effects of

Figure VI-3

The effect of pentobarbitone on glutamate responses

Serial measurements made during a single experiment. 2 applications of 2.5×10^{-4} M pentobarbitone were made, 25min apart. In this cell pentobarbitone caused hyperpolarisation (3.5mV and 4mV) as well as a depression of the responses to standard pulses of glutamate (200nA).

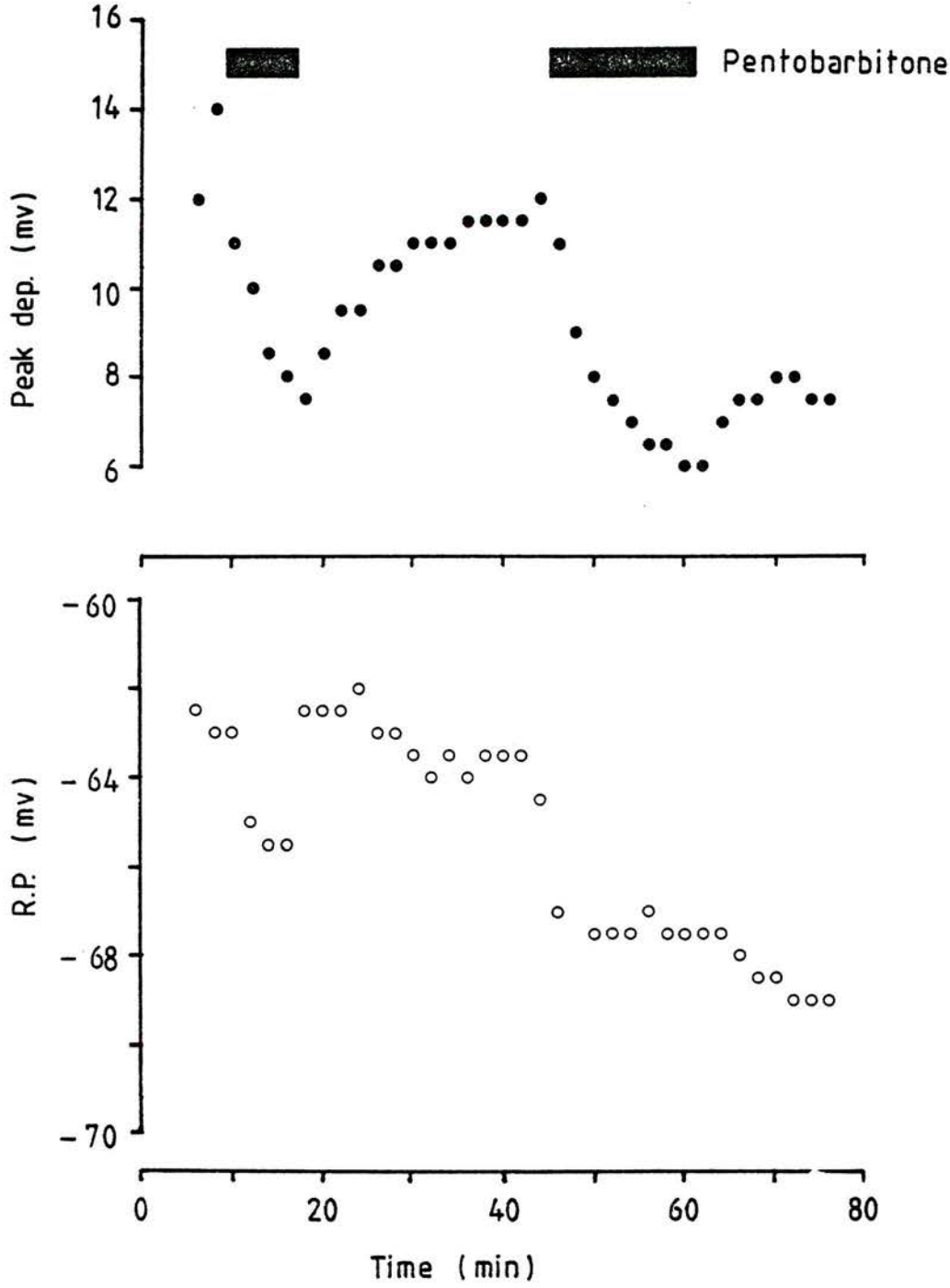

Pentobarbitone
Peak dep. (mv)
16
14
12
10
8
6
R.P. (mv)
-60
-64
-68
-70
0
20
40
60
80
Time (min)

Figure VI-4

Effects of pentobarbitone and ketamine on glutamate responses

A: Pentobarbitone (3×10^{-4}M) reduced both the depolarisation and the change in input resistance produced by standard pulses of glutamate. Exposure time: 3min Wash time: 8min

Iontophoretic pulse: 600nA, 2s

Hyperpolarising pulses: 1nA

B: Ketamine (3.7×10^{-4}M) had only a small depressant effect.

Exposure time: 8min Wash time: 10min

Iontophoretic pulse: 20nA, 5s

Hyperpolarising pulses: 1nA

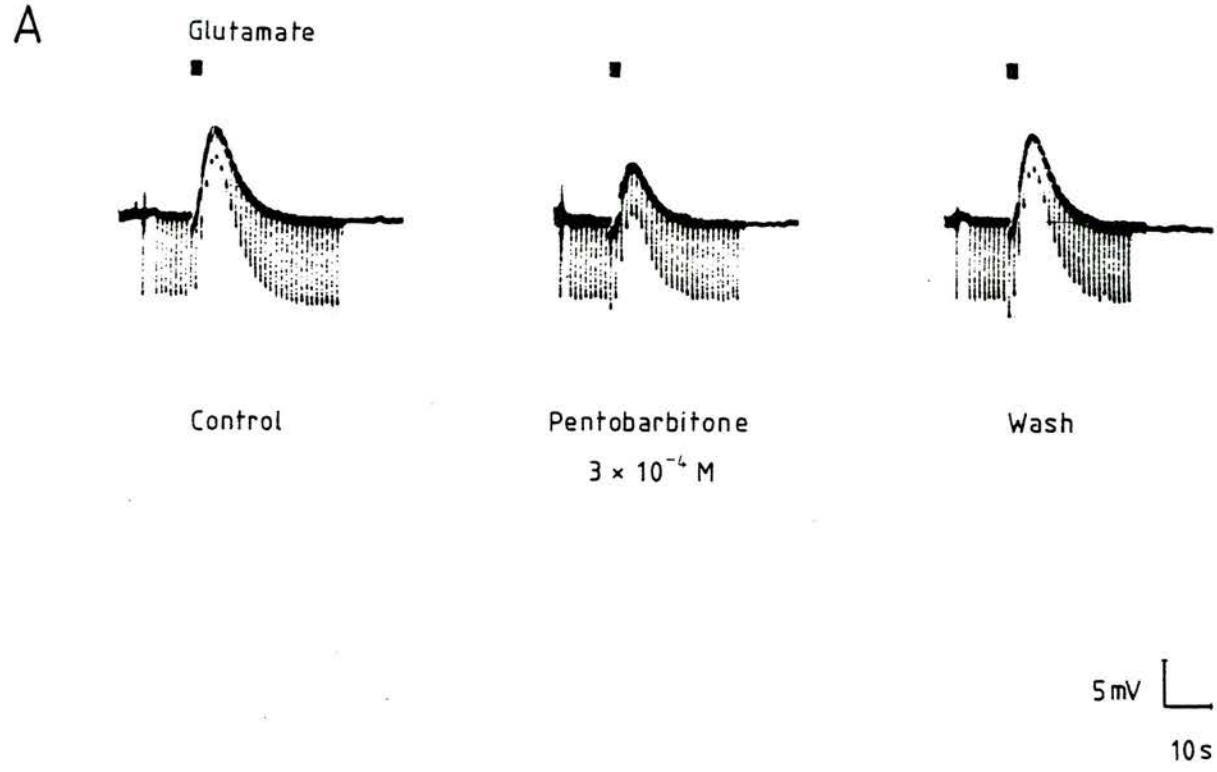

A
Glutamate
Control
Pentobarbitone
3 × 10⁻⁴ M
Wash
5 mV
10 s
B
Glutamate
Control
Ketamine
3·7 × 10⁻⁴ M
Wash

Figure VI-5

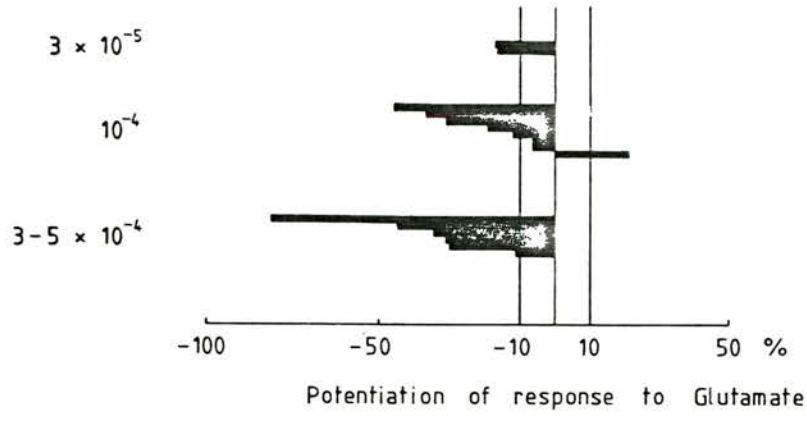
Effects of pentobarbitone and ketamine on responses of individual cells

Area enclosed by the envelope of the response measured. Each bar represents the % potentiation of the control measurement for a single cell. Changes of less than 10% were not considered important.

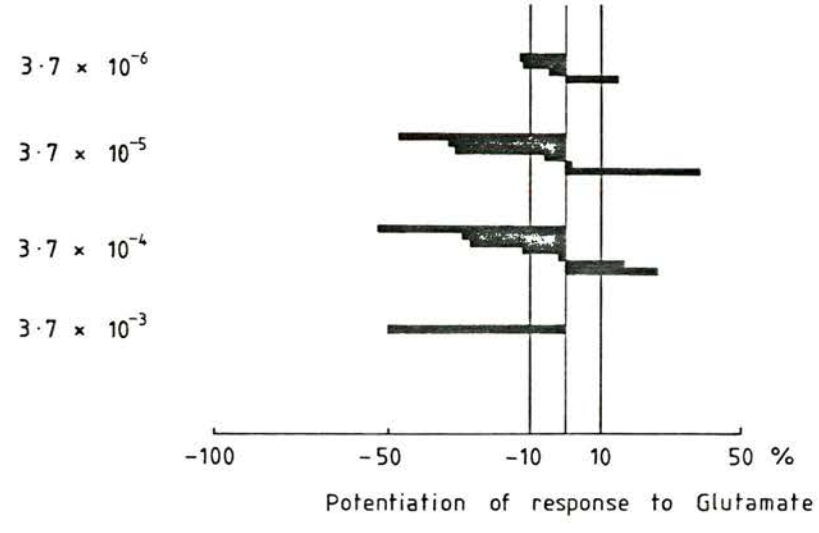
Pentobarbitone (upper bar chart): all concentrations depressed the responses and the depression appears to be dose-related.

Ketamine (lower bar chart): no concentration produced a consistent change, but the response was depressed in the majority of cells when the concentration exceeded 10^{-5} M.

Pentobarbitone (M)



Ketamine (M)



higher concentrations of ketamine were no greater, nor were they more consistently seen.

Alphaxalone

As with ketamine, the variation between experiments resulted in all effects being statistically non-significant (Appendix II). However, Figure VI-7 shows that depressant effects predominated and were on balance greater in the presence of alphaxalone than when the commercial vehicle was presented alone. There was no clearly dose-related effect and the biggest depressions were seen during exposure to the lowest concentration of alphaxalone.
Records from a single experiment are shown in Figure VI-6A, but these cannot be considered typical in view of the variable results.

Metomidate

The number of experiments carried out with metomidate was small, but the summary figure (Figure VI-7) shows that, as with ketamine and alphaxalone, there was considerable variation between the results. Increasing the experimental numbers was likely merely to emphasize this, as in the case of the clinically relevant concentration of alphaxalone ($1\text{-}3 \times 10^{-5}$ M, Figure VI-7). The only cell in which a potentiated response was seen was one exposed to the lowest concentration of metomidate, and depressed responses were seen only at supra-anaesthetic concentrations. It is notable that in two out of three tests the depression by 1.8×10^{-3} M metomidate did not exceed 15% (contrast the total elimination of GABA and glycine responses; Figures V-8 & 9). Records taken from an experiment in which the effects of metomidate were only slight are

Figure VI-6

Effects of alphaxalone and metomidate on glutamate responses

Alphaxalone (A) depressed the glutamate responses slightly but metomidate (B) had little effect.

A: sample responses after 11min exposure to alphaxalone; 27min wash.

Iontophoretic pulses: 500nA, 5s

Hyperpolarising pulses: 0.9nA

B: sample responses after 7min exposure to metomidate; 16min wash.

Iontophoretic pulses: 400nA, 4s

Hyperpolarising pulses: 1nA

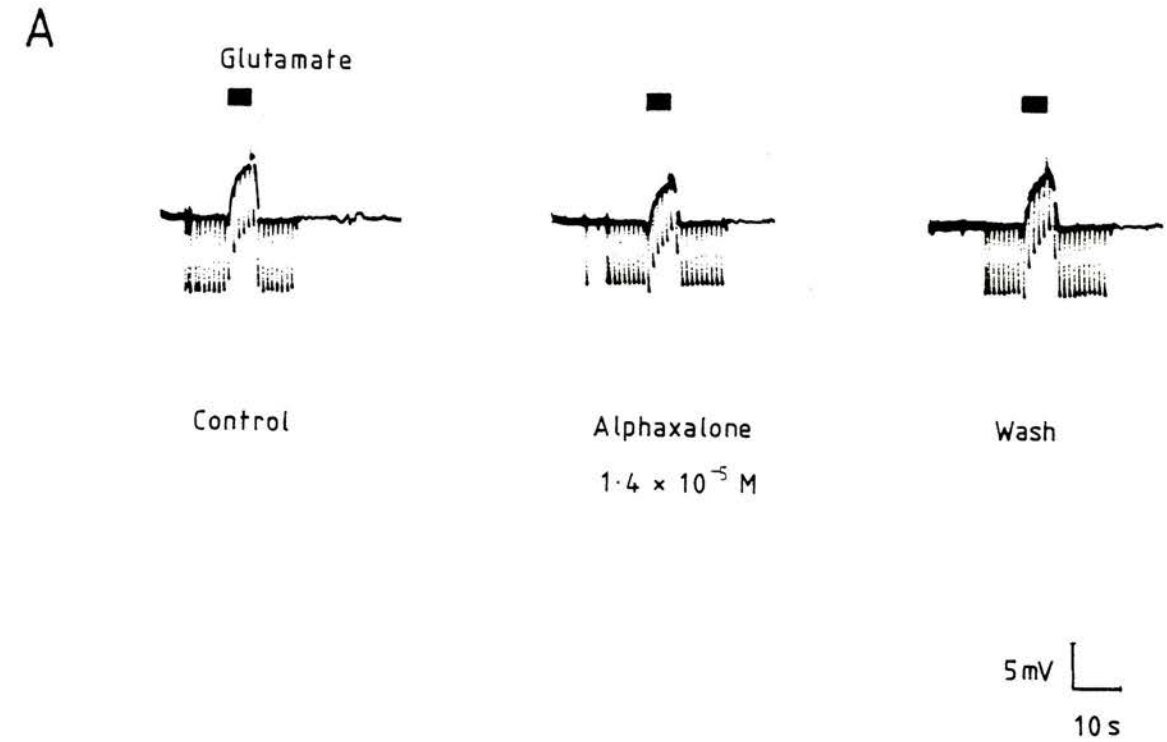

A
Glutamate
Control
Alphaxalone
1·4 × 10⁻⁵ M
Wash
5 mV
10 s
B
Glutamate
Control
Metomidate
1·8 × 10⁻³ M
Wash

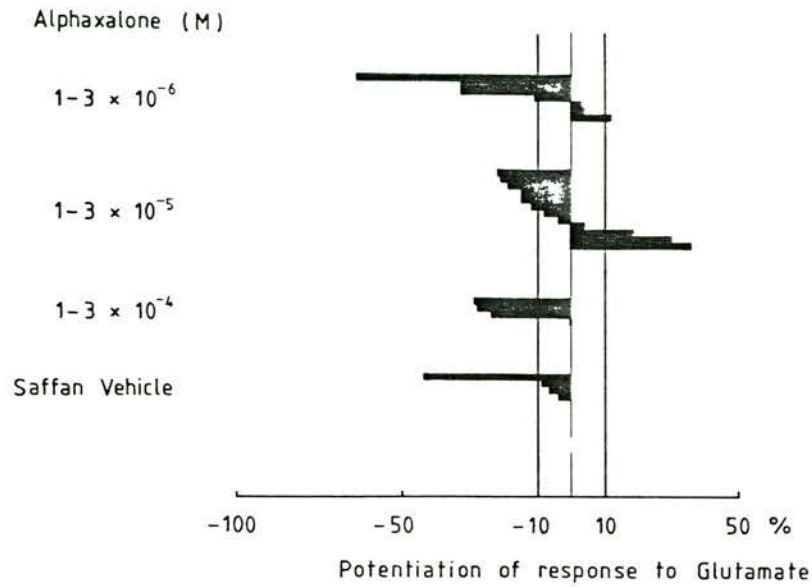
Figure VI-7

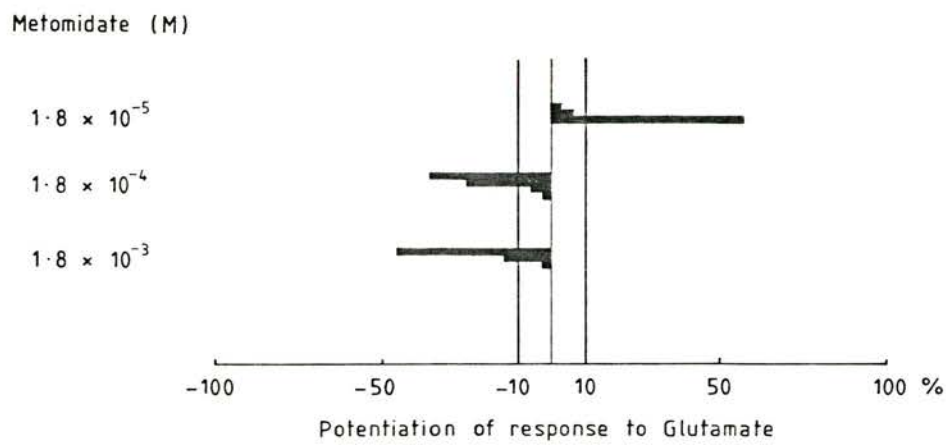
Effects of alphaxalone and metomidate on responses of individual cells

Area enclosed by the envelope of the response measured. Each bar represents the % potentiation of the control measurement for a single cell. Changes of less than 10% were not considered important.

Alphaxalone (upper bar chart): note the variability of the effects; only the highest concentration ($1\text{-}3 \times 10^{-4}$ M) depressed the responses of a majority of cells (3 out of 4).

Metomidate (lower bar chart): no concentration produced a consistent effect, and only 1 test in 10 showed an increased response.


Alphaxalone (M)
1-3 × 10⁻⁶
1-3 × 10⁻⁵
1-3 × 10⁻⁴
Saffan Vehicle
-100
-50
-10
10
50 %
Potentiation of response to Glutamate


Metomidate (M)
1·8 × 10⁻⁵
1·8 × 10⁻⁴
1·8 × 10⁻³
-100
-50
-10
10
50
100 %
Potentiation of response to Glutamate

displayed in Figure VI-6B. When the results from all cells were combined, no concentration of metomidate had a statistically significant effect (Appendix II).

The effect of agonist dose on the anaesthetic effects (not illustrated)

In no case did variation in the pulse length have any detectable effect on the influence of the anaesthetics. This was not surprising in view of the inconsistent effects of three of the drugs.

The effect of substance P on bulbar Müller cells

Injection of substance P into the perfusate (solution C) had a dramatic excitatory effect on the bulbar cells that was manifested as periodic depolarisations of 5-10mV accompanied by excessive synaptic activity and bursts of action potentials (Figure VI-8A). This response represented an accentuation of the normal behaviour seen in some cells; it was also very similar to behaviour seen in the presence of a low dose of ketamine (Figure IV-3). Elevation of the extracellular [Mg^{2+}] to 15mM eliminated all synaptic activity, leaving a response to substance P which consisted of a small sustained depolarisation rather than repeated depolarising surges (Figure VI-8B).

Discussion

Responses to Glutamate

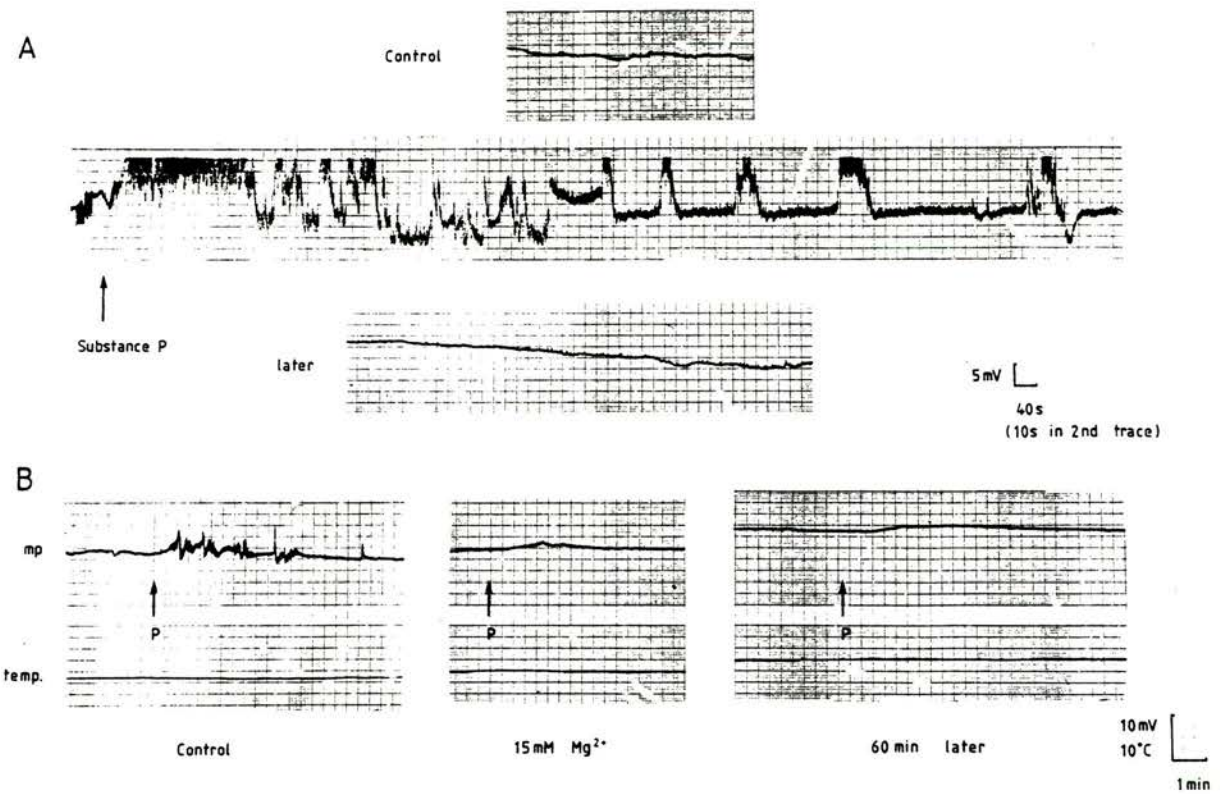
Whereas all cells tested responded to GABA and glycine (Section V), an unexpectedly high proportion gave no detectable response to glutamate. This finding is striking because glutamate is widely distributed in the vertebrate CNS (Hebb, 1970) and, in an earlier

Figure VI-8

The excitatory effect of substance P on bulbar Müller cells

A: injection of substance P (0.1ml of 10^{-3}M solution) into the perfusion line caused a short latency excitatory effect which consisted of synaptic potentials superimposed on a depolarisation lasting 1min, followed by phasic depolarisations at progressively increasing intervals. Note that the depolarising potentials have been truncated by the pen-recorder; in fact action potentials were fired at the height of the activity.

B: the direct efect of substance P on Müller cells: in the presence of 15mM Mg^{2+} the synaptic potentials have been abolished but there remains a small (1.5mV) depolarising response to a bolus injection of substance P. The temperature trace is displayed to demonstrate that the depolarisations cannot be ascribed to fluctuations in temperature.


A
Control
Substance P
later
5mV
40s
(10s in 2nd trace)
B
mp
temp.
P
P
P
Control
15mM Mg2+
60 min later
10mV
10°C
1min

study using the lamprey preparation, there was no mention of unresponsive cells (Matthews & Wickelgren, 1979b). Whilst rapid receptor desensitisation could account for some cells' not responding to glutamate, it should have affected the latter study also. Curtis, Phillis & Watkins (1960) found no evidence that spinal neurones became desensitised to glutamate. Possibly the position of the iontophoresis electrode in relation to a Müller cell was more critical in the case of glutamate. This would be expected if, for example, the glutamate receptors were not distributed throughout the cell membrane but were localised to synaptic regions or even to synapses on particular processes. To resolve the question, experiments using an improved optical system are required, so that the position of the electrode tip is known more precisely. When an electrode was positioned, it was moved until the response was optimal or, in experiments where the inhibitory transmitters were used as well, at least adequate. In this context it was notable that few cells showed a response when the micropipette was filled with 0.5M glutamate instead of 2M (see Section II), this contrasting with the satisfactory responses obtained using 0.5M solutions of GABA and glycine. However, no conclusions can be drawn without a knowledge of the transport numbers of the three amino acids in the solutions used.

The shapes of the responses observed lay between two extremes; the one with much greater depolarisation, and the other a smaller response which could not greatly be increased by lengthening the iontophoretic pulse (i.e. applying a larger dose of glutamate). It is possible that these were distinct responses with different

reversal potentials, but reversal potentials were not measured in these experiments. The associated decrease in input resistance ranged from 65% to undetectable and was not correlated with the change in membrane potential. The change did not exceed 15% in 28 out of 45 cells, and lay between 20% and 30% in 11 cells. The suggestion that more than one receptor type was concerned receives some support from previous observations in other preparations. For example, Lambert & Flatman (1981) reported that prolonged application of glutamate to lumbar motoneurones in cats caused depolarisation and a slowly developing increase in membrane conductance, and that whereas this conductance increase was always reduced by barbiturates, the depolarisation was not reduced in all experiments. It is interesting that, by grouping the excitatory amino acids according to the nature of the response elicited from motoneurones, Lambert, Flatman & Engberg (1981) produced a classification which differed in some respects from the pharmacological classification discussed previously. For example, L-glutamate and L-aspartate were alike in causing an increase in conductance and a depolarisation fast in onset and offset, whereas NMDA caused a marked fall in conductance coupled with a much slower depolarisation. Responses to kainate were unlike those to any other amino acid tested. Matthews & Wickelgren (1979b) measured a conductance increase only when glutamate was applied to the somata of lamprey bulbar cells: although depolarisation was also recorded following application to the axons. These authors suspected that axon depolarisation was the result of passive spread of current from cells that were coupled electrically; both electrical and chemical

afferent synapses have been described on Müller axons (Rovainen, 1967).

The almost universal excitatory effects of glutamate and aspartate have been used as arguments for the presence of extra-synaptic receptors for these amino acids on most cells (Nistri & Constanti, 1979). If this be so, then an added uncertainty is the extent to which the pharmacological properties of synaptic and extrasynaptic glutamate receptors differ. By virtue of their position, extrasynaptic receptors must be more accessible to exogenous amino acids, and it is possible that the big responses, seen only rarely, represent the responses of synaptic receptors. However, the iontophoretic currents that elicited large amplitude depolarisations were often much smaller than those required to produce a small "squat" response in other cells. This might argue for the former's being extrasynaptic. Such differences were observed between cells in a preparation as well as between preparations so they cannot have been due merely to differences between electrodes. There was no information as to the quantity of glutamate delivered when a given iontophoretic current was passed by a particular pipette (see Section II).

Allusion has been made already to the existence of multiple glutamate receptor types, and to the fact that they differ in their sensitivities to magnesium. Evidently the effects of the magnesium concentration used in the present study varied between preparations, since synaptic potentials in response to glutamate were not always eliminated.

The effects of anaesthetics on glutamate responses

The most striking aspect of these experiments was the contrast with the results of experiments with GABA and glycine. Only two drugs showed any consistent effect, and only with pentobarbitone was the depression statistically significant and apparently dose-related. The results with metomidate were particularly dramatic as indicated already, and suggest that the depressant effects of high concentrations of all anaesthetics on the inhibitory responses cannot be the result of an entirely non-specific neuronal depression.

The depressant effect of pentobarbitone is consistent with earlier reports of barbiturate depression of responses to excitatory amino acids (e.g. Krnjević & Phillis, 1963; Crawford & Curtis, 1966; Johnson, Roberts & Straughan, 1969; Richards & Smaje, 1976; Nicoll & Wojtowicz, 1980). A similar depressant effect of alphaxalone, described by Richards & Smaje (1976) in the prepyriform cortex, was not found in the lamprey preparation. Barker & Ransom (1978a) in studies on cultured mouse neurones found the sensitivities of the GABA and glutamate responses to pentobarbitone to be about equal. Pentobarbitone depressed the glutamate responses in the absence of an increase in membrane conductance, and the interaction appeared to be one of non-competitive antagonism.

The suggestion that ketamine also has some glutamate inhibitory effect is consistent with the report (Anis, Burton & Lodge, 1982) that ketamine selectively inhibits NMDA responses and has a lesser depressant effect on responses to glutamate, quisqualate and kainate. The capricious nature of the effect in the lamprey may be

connected with the variable effect of 15mM Mg^{2+}, since magnesium has been shown selectively to depress NMDA-preferring receptors (Ault et al, 1980). It should be noted also that Anis et al were using sub-anaesthetic doses of ketamine (2.5-10mg/kg) or, when ketamine was applied by iontophoresis, currents smaller than those which affected GABA and glycine responses (see Section V).

As to the mechanism of these effects there is little relevant published evidence. However, Minchin (1981) in biochemical uptake studies found that whereas pentobarbitone (but neither alphaxalone nor ketamine) inhibited potassium-stimulated GABA and aspartate release from the cortex, no drug tested appreciably affected either the spontaneous release or the uptake of aspartate. Moroni et al (1981) reported that pentobarbitone did not reduce glutamate release from the cortex.

Implications for the anaesthetised animal

Consideration of the implications of these results for an anaesthetised animal requires a synthesis of the results of Sections IV, V & VI, and is deferred to Section VII. However, a few comments are appropriate here. Firstly, the depressant effects of pentobarbitone on the bulbar Müller cells, part of an inhibitory pathway, is consistent with the finding of Frank & Ohta (1971) that several anaesthetics, including pentobarbitone, blocked the reticulo-spinal inhibitory pathway which inhibits segmental reflex potentials in decerebrate cats, and this at concentrations no greater than those attained during surgical anaesthesia. Secondly, the absence of depression of glutamate responses by alphaxalone and metomidate

may relate to extraneous non-purposive movements and muscle twitching reported by clinicians to occur during anaesthesia with these drugs: such movements are likely to be central in origin (Granit, Holmgren & Merton, 1955) and, in any case, the effects of alphaxalone at the neuro-muscular junction are inhibitory (Pennefather, Puil & Quastel, 1980).

Effects of substance P

At this preliminary stage of the investigation, little can be said beyond the observation that substance P excited the bulbar cells. The phasic nature of the excitement produced was characteristic and it is tempting to relate it to the phasic activity recorded by Buchanan & Cohen (1982) from interneurones, motoneurones and slow muscles of the lamprey. Whilst the synaptic potentials evidently arose elsewhere in the CNS, there was also an observable direct effect on the bulbar cells in the presence of 15mM Mg^{2+} to block synaptic transmission: synaptic potentials were not then observed but there remained a sustained depolarisation that was small (about 1mV) yet reliably produced. This peptide has been shown to have excitatory effects in many parts of the vertebrate CNS.

SECTION VII

Epilogue

> ...whenever we attempt to combine our scattered physiological facts, we are stopped by the want of numerous intermediate analogues: and so loosely connected or so independent of each other, are the different series of phaenomena, that we are rarely able to make probable conjectures, much less certain predictions concerning the results of new experiments...
>
> Davy (1800)

Davy's remark, made in a different connection, sums up the problem of extrapolating from the results presented here to the complex and progressive changes which occur in the brain of a mammal during anaesthesia. Observations on the interference by sundry anaesthetics with synaptic functioning and with the responses of cells to transmitters abound and the investigation of anaesthetic actions in the brain has not so far been tackled in a coordinated manner. When the question of the general applicability of results obtained in the lamprey is addressed, several supplementary questions demand answers, among them:

i) are the receptor/ionophore complexes in the membranes of lamprey cells identical to those in equivalent cells in mammals?

ii) Are the receptor/ionophore complexes in synaptic and extrasynaptic sites within the membrane of a single cell identical in their responses? Inevitably in experiments in which iontophoresis or bath application of transmitter has been employed, there is no control over the exposures of individual receptors.

iii) Are the receptor/ionophore complexes the same in different regions of the brain, or are there fundamental differences which render any comparison of their responses to anaesthetics meaningless?

iv) Is the effect of an anaesthetic when the natural transmitter is acting on its sub-synaptic receptor similar to its effect when an exogenous transmitter is applied generally to the membrane?

v) Some of the more recent elegant studies have used cells in culture (e.g. Barker & Ransom, 1978a,b; Barker, McBurney & MacDonald, 1982): are there any fundamental differences between the functioning of these cells and that of cells in situ?

To such questions there are as yet few answers; the investigation of anaesthetic-transmitter interactions is still at the descriptive stage. The lamprey preparation enables anaesthetic modulation of the responses of single identified cells to be assessed, and thus serves as a model for one link in the complex chain of events occurring in the brain. It is useful in the sense described by Beament (1960) in his discussion of the application of physical models in biology - the model is simpler than the original but is in similar circumstances to those of the original.

My findings are restated in summary form in Table VII-1. Some comment on the likely implications of the effects described is now possible in the light of the three series of experiments.

Pentobarbitone

Sub-anaesthetic concentrations potentiate responses of Muller cells to GABA whilst having little effect on those to glycine and glutamate. Anaesthetic concentrations in addition have a depressant effect on glutamate responses and on the level of spontaneous synaptic activity. Since glycine rather than GABA is thought to be the inhibitory transmitter acting on Müller cells under normal

Table VII-1

Summary of Results

Anaesthetic		GABA			Glycine			Glutamate
	Conc.	Resist.	Area	A/P	Resist.	Area	A/P	Peak
Pentobarbitone	High	-	-		0	0		-
	Low	0	+	+	0	0	0	-
Ketamine	High	0	+	+	-	-		-
	Low	0	?+	?+	0	0	0	0
Alphaxalone	High	-	-		-	-		?-
	Low	-	-		0	0		0
Metomidate	High	-	-		-	-		0
	Low	0	0		0	0		0

Symbols represent: + potentiated (?+ uncertain); - depressed (?- uncertain); 0 no change
Missing values were not tested but showed no change on visual inspection.
"High" and "Low" concentrations of anaesthetics are thus designated in relation to those likely to be encountered during clinical anaesthesia.
A/P denotes the calculated ratio Area/Peak (see Section II).

conditions, no conclusions about the relative importance of pre- and postsynaptic effects can be drawn. The role of the GABA receptors on these cells is not known, any more than is that of extra-synaptic GABA receptors elsewhere in the nervous system, nor is the origin of the GABA to which they normally respond. However if the GABA is of synaptic origin, it would be predicted that potentiation of the cells' responses to GABA would in part offset the implied depressant effect on presynaptic function. Pentobarbitone had no effect likely to increase activity in the Müller axons; all effects described would be expected to reduce motor responses to stimulation and so promote muscle relaxation.

Ketamine

The increase in the spontaneous activity of Muller cells seen during exposure to low concentrations of ketamine (Section IV) was not matched by an equivalent change in their responses to exogenous amino acid transmitters (Sections V & VI). As with pentobarbitone, the most dramatic effects were those on GABA responses; and their significance must depend on the extent to which the extra-synaptic receptors are important in themselves and respond to anaesthetics in a manner similar to that of synaptic receptors elsewhere in the CNS. I observed no potentiation of glutamate responses resulting from exposure to low concentrations of ketamine but have tested no other putative excitatory transmitter. In view of the similarity of the activity recorded in 3.7×10^{-7} M ketamine to that caused by bath application of substance P, it may be that peptide transmitters are involved in such excitatory reactions. Anaesthetic concentrations of ketamine depressed glycine responses more than those to glutamate

and IPSPs were more susceptible to block than were EPSPs. Both results suggest that there will be resultant disinhibition of these motor cells and so, potentially, exaggerated motor responses to stimulation. This presupposes that the other central actions of ketamine do not interfere with the afferent input to the cells, and such a supposition may not be valid since ketamine is regarded as a dissociative anaesthetic. However, the results are consistent with the high level of muscle tone found clinically during ketamine anaesthesia.

Alphaxalone and Metomidate

Such complete block of responses to inhibitory amino acids as was seen with alphaxalone ($>10^{-5}$M) and metomidate (10^{-3}M) should be reflected in reduced transmission at synapses involving these receptors. The relative lack of effect on the glutamate responses was marked with these drugs, also, and would reinforce the disinhibiting effect. The results are consistent with the recorded occurrence of non-purposive movements during anaesthesia with these drugs, particularly during the recovery period. When the anaesthetic concentration is higher, perhaps at levels present during surgery, the presynaptic depressant effect implied by the abolition of EPSPs and IPSPs probably offsets the disinhibition. The effects of the two drugs were similar, but the significance of the observation that those of metomidate were only apparent at rather high concentrations is not clear. However, it would seem prudent to avoid unnecesssary stimulation when either drug is used clinically.

It was a general finding that the IPSPs were more susceptible to anaesthetic blockade. If IPSPs are in part concerned with discriminative inhibition, as has been suggested by Andersen et al (1980), this may contribute to the reduction in the fine control of motor responses commonly found during induction and recovery from anaesthesia.

This investigation has little to add to the current theories of anaesthesia. Any proposed general mechanism of anaesthesia must be shown to involve effects that are both necessary and sufficient for anaesthesia. The only general conclusion that can be drawn from the present experiments is that none of the effects described is necessary, much less sufficient, for anaesthesia. Provided that amino acid receptors on the bulbar Müller cells in the lamprey brain are acceptable as a model for synaptic amino acid receptors in the mammalian brain, this means that it is not necessary for anaesthesia that the effects of any of the transmitters glutamate, GABA and glycine on receptors of the types present on Müller cells be either potentiated or inhibited. The argument will be strengthened if similar results are obtained with other preparations, and if experiments measuring anaesthetic effects on responses to endogenous transmitters are carried out in parallel. The latter will demand control of, or compensation for, possible effects of the anaesthetics on presynaptic mechanisms. Even were this possible, the objection would remain that anaesthesia is a clinical state (or states) which cannot fairly be represented by easily measurable effects on a single cell.

The response feature which I have measured in Section V, the

input resistance, is closer to the fundamental response than are such features as changes in membrane potential or firing frequency. Changes in membrane potential depend on the resting membrane conductance, the induced change in conductance and the driving force on the permeant ions, and have been classed as second order effects (Werman, 1969). Justification of the use of changes in firing frequency depends on the dubious assumption that the membrane potential is linearly related to the firing frequency. Ideally, the quantity measured should be directly proportional to the fundamental response. With cells of complex geometry, such as the bulbar Müller cells, calculation of the membrane conductance from the input conductance is not simple and has not been attempted. This does not affect my results adversely: it would have been more important had I attempted a quantitative treatment of the kinetics of the responses (see discussion by Werman, 1969). Moreover, because the experiments were not conducted under voltage clamp, membrane potentials were measured, not membrane currents, so the quantity measured was an inverse function of conductance. To calculate the conductance from the potential measurements would thus be to emphasise unduly the measurement errors when the resting conductance was high. I have therefore chosen to display the measurements of input resistance rather than their reciprocal, the membrane conductance. It is unfortunate that the errors were necessarily greater at the peaks of the GABA and glycine responses. Notwithstanding this, in most previous studies of anaesthetic effects on single neurones in the CNS it is firing frequency that has been measured.

Mention has been made already of the different CNS states induced by anaesthetic agents (Sections I & IV, pp.24 & 104). Anaesthetic drugs have a range of pharmacological effects and some actions, only, are common to all drugs. Anaesthetics frequently depress respiratory and cardiovascular function as well as consciousness, and induce varying degrees of analgesia and muscle relaxation. The relative potency in respect of these different actions is not the same for all drugs. One way of achieving specificity of action would be for drug-specific effects on the various neurotransmitter systems to exist; and in support of this suggestion is the growing body of evidence that anaesthetics interact stereo-specifically with certain proteins in such a way as to affect their functional properties. For example Baker & Shapira (1980) have shown that some anaesthetics increase the emission of light by the calcium-sensitive protein aequorin in the presence of constant ionised calcium levels. If their suggestion that other calcium-binding proteins may behave in a similar manner proves true, alterations in presynaptic function are likely. It is equally possible that transmitter receptor proteins may bind anaesthetics stereo-specifically and that their properties may change accordingly. Stereo-isomers of three of the anaesthetics I have used (pentobarbitone, ketamine and alphaxalone) are known to differ in their anaesthetic properties. So far as I am aware isomers of metomidate have not been tested, but those of etomidate have been shown to have different anaesthetic potencies (see Section I).

As an illustration of the difficulty of making extrapolations from single cell studies, I shall discuss in some detail an apparent

conflict between some of my results and those of a study already cited (Scholfield, 1980; see Section V) and show that a degree of reconciliation of the two studies is possible. Scholfield's study concerned the effects of anaesthetics on recurrent inhibition in the guinea-pig olfactory cortex. Recurrent inhibition was shown to be prolonged and intensified by a variety of anaesthetics including pentobarbitone and alphaxalone (prolongation of IPSCs at least x10) and ketamine (prolongation up to x2). The effect of alphaxalone and the relative lack of effect of ketamine are particularly surprising in view of my results.

Scholfield stimulated the lateral olfactory tract electrically and recorded intracellularly from cortical cells. The response feature measured - a prolonged after-depolarisation associated with an increase in membrane conductance (IPSC) - is thought to result from activation of an inhibitory interneurone (Pickles & Simmonds, 1978; Scholfield, 1978). The IPSC therefore represents the result of transmission at a minimum of two synapses, and the anaesthetic effects recorded may have arisen at one or more of several sites. In the present study I have applied transmitters directly to the Muller cells, so any effect of the anaesthetics on presynaptic GABA or glycine receptors should not be apparent. The time course of the IPSCs in the olfactory cortex (200-500ms) was considerably longer than the average duration of an IPSP recorded from a lamprey Müller cell (40ms).

The identities of the transmitters at each site in the olfactory cortex have yet to be firmly established but there is

evidence that both GABA and aspartate are involved (Pickles & Simmonds, 1976; Collins, 1979). There are also indications that GABA acts here on two or more pharmacologically distinct receptor types. Thus both bicuculline, a GABA antagonist at postsynaptic sites, and baclofen, an agonist at presynaptic GABA receptors (Hill & Bowery, 1981) block the IPSC and its field potential correlate, the "I" wave (Pickles & Simmonds, 1978; Cain & Simmonds, 1982; Scholfield, 1982,1983). Baclofen is likely to have reduced transmitter release at both synapses in the chain leading to the IPSC whereas bicuculline probably blocked the inhibitory effect of GABA released from the inhibitory interneurone. Pickles (1979) invoked different postsynaptic GABA receptor types on the somata and dendrites of cortical cells to explain an anomalous action of bicuculline in potentiating the effects of high doses of GABA. Insofar as their pharmacology is understood, the GABA receptors on lamprey Muller cells seem to be of the conventional bicuculline-sensitive type (Martin, 1978, 1979a; Matthews & Wickelgren, 1979a).

Where the anaesthetics were acting in Scholfield's study cannot be certain, but extrapolations from my results (Table VII-1) lead to some suggestions:

Pentobarbitone has been shown to increase the "I" wave (Pickles & Simmonds, 1978) and is known to potentiate the effects of GABA in many preparations (see below). It is likely that the effect of GABA released from the inhibitory interneurone was increased. The result is the opposite of that predicted were pentobarbitone to potentiate GABA's actions at presynaptic receptors. Scholfield's peak effect was recorded at sub-anaesthetic concentrations (2×10^{-5} M). It may

be that at higher doses depressant effects, such as were responsible for the loss of spontaneous synaptic activity in Section IV, outweigh the GABA potentiation.

Ketamine may have exerted only a moderate potentiation of the IPSC in Scholfield's study because it not only potentiates GABA actions, it also antagonises the effects of aspartate (Anis, Burton & Lodge, 1982; see Section VI). The transmitter exciting the interneurone has yet to be identified but release of aspartate has been demonstrated following stimulation of the lateral olfactory tract (Collins, 1979). As with pentobarbitone, my observation of reduced spontaneous activity at higher concentrations ($>10^{-4}$ M) suggests that any potentiation effect seen at lower doses in a polysynaptic pathway would be attenuated by higher doses.

Alphaxalone markedly increased the IPSC at concentrations below those required to antagonise GABA in the lamprey. It is possible that an antagonistic effect was outweighed by facilitation of transmission at the excitatory synapse on the inhibitory interneurone - Scholfield reported no reduction of the EPSP at concentrations below 10^{-5} M. I have shown in Section IV that low concentrations of alphaxalone increased the frequency of EPSPs recorded from Müller cells and, although I have found no potentiation of glutamate effects (Section VI), I did not test aspartate. An added consideration is that the concentrations of alphaxalone in Scholfield's solutions may have been lower than the figures quoted - the steroid has low solubility and was dissolved (or "dispersed") directly in Krebs solution by vigorous shaking.

Scholfield did not test metomidate or etomidate. By analogy with alphaxalone, my results predict that metomidate would potentiate the IPSC. The potentiation might be less pronounced than with alphaxalone, there being less increase in EPSPs recorded in Section IV. Since metomidate had little effect on transmitter responses at concentrations below 10^{-3} M, reduced potentiation would be expected only at high concentrations. However, I did not test aspartate, which is thought to be one excitatory transmitter in the olfactory cortex. Predictions of this nature can only be speculative - Scholfield's observations concerned the responses of synaptic receptors to their natural transmitter in a polysynaptic pathway. Quite apart from these differences, the region of the brain and the species of animal were also different.

Another point of difference between the studies is the much greater effect Scholfield observed on passive membrane properties. I have found nothing comparable to the 40% reduction in input resistance which he obtained with alphaxalone concentrations over 10^{-5} M, or to the 90% reduction due to pentobarbitone (2×10^{-4} M to 10^{-3} M); nor have I detected an effect of the higher concentrations of ketamine to increase the input resistance.

There is no necessity that anaesthetics should exert identical effects on different membranes or GABA receptors in the brain so the disparities in the results do not immediately rule out the lamprey preparation as a useful model for the equivalent region of the mammalian CNS. So long as the limitations of the preparation are clearly appreciated its many practical advantages, discussed in Section I, make the lamprey eminently suitable for the study of the

pharmacology of anaesthetics. Not only are experiments of the type reported herein feasible using identified cells, but it seems likely that the use of more sophisticated techniques, such as the patch clamp, will be possible also. These will give some insight into the mechanisms underlying the observed effects.

I conclude, with Davy (1800):

> ... from the difficulty of discovering every possible mode of examination, and from the modification of perception by the state of feeling, it appears nearly impossible that all the relations of a series of phaenomena can be discovered by a single investigation, particularly when these relations are complicated, and many of the agents unknown. Fortunately for the active and progressive nature of the human mind, even experimental research is only a method of approximation to truth ... early experience has taught me the folly of hasty generalization...

and, with Porta (1561):

> ...if I have over-passed some things, or not spoken so properly of them, as I might; I know there is nothing so Beautiful, but it may be adorned; nor so Full, but it may be Augmented...

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APPENDIX I

The effects of anaesthetics on the responses to GABA and glycine

The numerical data from the experiments of Section V are tabulated in Table A-1. Each figure represents are the percentage change in response during application of an anaesthetic: mean ±s.e. Numbers of observations in parentheses.

Significant effects indicated (unpaired t-test): * $p < 0.05$;

** $p < 0.01$; *** $p < 0.001$.

Saffan vehicle dilution equivalent to 10^{-5} to 10^{-4}M.

Table A-1 The effects of anaesthetics on the responses to GABA and glycine

Anaesthetic	GABA			Glycine		
(M)	Resistance	Area	Area/Peak	Resistance	Area	Area/Peak
Pentobarbitone						
10^{-3}	-63 ±11(2)	-90 ±11(2)		+8 (1)	+3 (1)	
2-5 x 10^{-4}	+13 ±5(13)*	+25 ±10(11)*	+25 ±7(8)**	-15 ±9(9)	+4 ±16(8)	
10^{-4}	+9 ±11(6)	+36 ±17(8)	+71 ±30(8)*	+27 ±34(5)	-4 ±26(6)	
Ketamine						
3.7 x 10^{-3}	-4 ±15(2)	+108 ±49(2)	+233 ±0(2)***	-82 ±1(2)**	-86 ±3(2)*	
3.7 x 10^{-4}	+16 ±14(9)	+124 ±44(10)*	+78 ±17(10)**	-33 ±9(9)**	-23 ±13(10)	-8 ±12(9)
3.7 x 10^{-5}	+10 ±15(8)	+55 ±43(8)	+29 ±17(8)	-2 ±9(10)	-5 ±11(10)	+10 ±8(10)
Alphaxalone						
1-3 x 10^{-4}	-58 ±17(6)*	-69 ±30(4)		-39 ±9(4)*	-45 ±20(5)	
1-3 x 10^{-5}	-56 ±10(8)***	-74 ±5(8)***		-36 ±8(7)**	-45 ±11(7)**	
1-3 x 10^{-6}	-15 ±4(3)	-22 ±11(2)		-5 ±5(6)	+22 ±18(3)	
Saffan vehicle	-23 ±6(10)**	-21 ±9(10)*		-19 ±13(6)	0 ±7(6)	
Metomidate						
1.8 x 10^{-3}	-100 ±0(3)***	-100 ±0(3)***		-73 ±15(6)**	-77 ±17(6)**	
1.8 x 10^{-4}	-27 ±12(5)	-1 ±17(5)		-6 ±10(4)	+28 ±23(4)	
1.8 x 10^{-5}	-6 ±5(6)	+6 ±7(6)		+5 ±9(7)	+11 ±12(7)	

APPENDIX II

The effects of anaesthetics on the responses to glutamate

The numerical data from the experiments of Section VI are tabulated in Table A-2. Each figure represents the percentage change in response during application of an anaesthetic: mean ±s.e. Numbers of observations in parentheses.

N.B. Standard errors have been calculated and t-tests carried out only for the peak measurements.

Significant effects indicated (unpaired t-tests): * $p < 0.05$; ** $p < 0.01$ - 2-tailed test; \$ $p < 0.05$; \$\$ $p < 0.01$ - 1-tailed test. If it be assumed that no anaesthetic potentiates the effect of an excitatory transmitter, the 1-tailed test is appropriate.

Saffan vehicle dilution equivalent to 10^{-5} to 10^{-4}M.

Table A-2

The effects of anaesthetics on the responses to glutamate

Anaesthetic (M)	Glutamate response Peak	Area
Pentobarbitone		
$2\text{-}5 \times 10^{-4}$	-39 ±10(6)**	-41 (5)
10^{-4}	-17 ±8(8)$	-25 (7)
3×10^{-5}	-17 ±1(2)*$$	
Ketamine		
3.7×10^{-3}	-50 (1)	-51 (1)
3.7×10^{-4}	-12 ±10(7)	-6 (3)
3.7×10^{-5}	-13 ±13(6)	+1 (3)
3.7×10^{-6}	-4 ±7(4)	+8 (2)
Alphaxalone		
$1\text{-}3 \times 10^{-4}$	-20 ±7(4)$	-9 (2)
$1\text{-}3 \times 10^{-5}$	-4 ±6(12)	-3 (12)
$1\text{-}3 \times 10^{-6}$	-17 ±10(7)	-12 (7)
Saffan vehicle	-16 ±9(4)	-30 (4)
Metomidate		
1.8×10^{-3}	-21 ±13(3)	-20 (3)
1.8×10^{-4}	-18 ±8(4)	-17 (4)
1.8×10^{-5}	+22 ±17(3)	+15 (3)

Term (2) represents the variance between "cells", and term (5) the variance arising within "cells" (the "error").

The interaction represents the extent to which different neurones are affected differently by the anaesthetic. The significance of the interaction is assessed by testing (Cell x Test)/Error. Even if it is significant, as in this example, the anaesthetic "main" effect can validly be tested because the mean square representing the anaesthetic effect ("Test") contains the variance component of the interaction (see discussion by Sokal & Rohlf, 1973). The ratio calculated to assess the significance of the anaesthetic effect is Test/Error.

Table A-3

Table of Analysis of Variance for 5 neurones exposed to 10^{-4} M pentobarbitone

For each neurone (N) the areas of 2 control and 2 test responses to GABA have been used:

N= 1	Control	34.7	Test	33.55
2		28.3		50.7
3		35.3		78.6
4		77.75		93.25
5		13.5		21.2

The columns (control and test measurements) have been regarded as a fixed replicated variable; and the rows (neurones) as a random variable. Each cell of the above table represents the mean measurement of 2 responses.

Source of variation	df	Sum of Squares	Mean Square	F
1.Total	1	43589.78	43589.784	16.25
2.Cell	4	10727.78	2681.895	230.85***
3.Test	1	1540.01	1540.012	5.42
4.Interaction	4	1137.50	284.375	24.48***
5.Residue	10	116.17	11.617	

*** $p < 0.001$

To assess the statistical significance of an effect the quantity "F" was calculated, F being the "variance ratio" of the two populations considered. The ratios calculated were:

for the "main" (anaesthetic) row effect: Test/Interaction;

"interaction" Interaction/Residue.

The analysis was unsatisfactory in this particular study for reasons that are related to its design. In brief, the absolute magnitude of the anaesthetic effect on any individual cell was no greater than the absolute magnitude of differences between the responses of different cells. The "signal to noise ratio" was therefore poor and effects that were dramatic turned out to be insignificant (for example the depression of GABA and glycine responses by the highest concentration of metomidate). This variability in control response amplitude between cells could be eliminated by normalising the responses but only at the cost of removing all variance from the control readings and thereby invalidating the analysis. Part of the variation in the case of GABA and glycine responses was caused by the proximity of the resting membrane potential to the reversal potentials of the responses. Small changes in membrane potential were apt to produce marked alterations in the response, because there was a relatively large change in the driving force on the permeant ion (chloride). This was true particularly when the conductance was high, as at the peak of a response.

It is obvious from Tables A-4 to A-8 that in very many tests the interaction was highly significant. Comparison of the tables with Figures V-3,5,7,9 and VI-5 & 7 reveals that this was sometimes

the result of an anaesthetic's having opposite effects on different cells, but that in other cases all effects were in the same direction (e.g. 3.7×10^{-4} M ketamine tested against glycine-induced resistance changes). These alternatives could not be distinguished.

It was for all these reasons that Student's t-test was used in preference, applied to normalised data and with the null hypothesis that the mean % change in the response in the presence of anaesthetic did not differ from zero. The t-test is in fact a special form of one-way AOV in which there are no replicate values (only one figure in each sub-unit of the table).

Table A-4

AOV: The effect of pentobarbitone on GABA and glycine responses

Concentration		GABA			Glycine		
(M)	Measure	Main effect	Interaction	n	Main effect	Interaction	n
2-5 x 10^{-4}	Peak	<0.05	<0.001	14	NS	<0.01	12
	Area	NS	NS	11	NS	<0.01	8
	Area/Peak	<0.01	<0.025	8			
	Resistance	<0.05	<0.001	13	NS	<0.001	9
10^{-4}	Peak	NS	<0.001	5	NS	<0.001	8
	Area	NS	<0.001	5	NS	<0.001	6
	Area/Peak	<0.05	<0.001	8			
	Resistance	NS	<0.025	6	NS	<0.001	5

Table A-5

AOV: The effect of ketamine on GABA and glycine responses

Concentration		GABA			Glycine		
(M)	Measure	Main effect	Interaction	n	Main effect	Interaction	n
3.7×10^{-3}	Peak	NS	NS	2			
	Area	NS	<0.001	2			
	Area/Peak	NS	<0.01	2			
	Resistance	NS	<0.01	2			
3.7×10^{-4}	Peak	NS	<0.001	10	NS	<0.001	9
	Area	NS	<0.001	10	NS	<0.001	10
	Area/Peak	<0.01	<0.025	10	NS	<0.001	9
	Resistance	NS	<0.025	9	<0.05	<0.001	9
3.7×10^{-5}	Peak	NS	<0.01	8	NS	<0.001	10
	Area	NS	<0.001	8	NS	<0.001	10
	Area/Peak	NS	<0.001	8	NS	<0.001	10
	Resistance	NS	<0.001	9	NS	<0.001	10

Table A-6

AOV: The effect of alphaxalone on responses to GABA and glycine

Concentration		GABA			Glycine		
(M)	Measure	Main effect	Interaction	n	Main effect	Interaction	n
$1\text{-}3 \times 10^{-4}$	Peak				<0.025	<0.001	5
	Area				NS	<0.025	5
	Resistance				<0.05	<0.01	4
$1\text{-}3 \times 10^{-5}$	Peak	<0.001	<0.01	8	<0.01	<0.05	7
	Area	<0.001	<0.001	8	<0.05	<0.01	7
	Resistance	<0.001	<0.001	8	<0.01	<0.05	7
$1\text{-}3 \times 10^{-6}$	Peak				NS	NS	5
	Resistance				NS	NS	6
Saffan vehicle	Peak	NS	<0.001	10	NS	<0.05	6
	Area	NS	<0.001	10	NS	NS	6
	Resistance	<0.01	<0.001	10	NS	<0.025	6

Table A-7

AOV: The effect of metomidate on responses to GABA and glycine

Concentration		GABA			Glycine		
(M)	Measure	Main effect	Interaction	n	Main effect	Interaction	n
1.8×10^{-3}	Peak	NS	<0.01	3	<0.01	<0.001	6
	Area	NS	<0.001	3	<0.025	<0.001	6
	Resistance	<0.01	NS	3	<0.025	<0.001	6
1.8×10^{-4}	Peak	NS	<0.025	5	NS	NS	4
	Area	NS	<0.05	5	NS	<0.001	4
	Resistance	NS	<0.001	5	NS	<0.05	4
1.8×10^{-5}	Peak	NS	<0.05	6	NS	<0.05	7
	Area	NS	<0.01	6	NS	<0.001	7
	Resistance	NS	NS	6	NS	NS	7

Table A-8

AOV: The effects of anaesthetics on responses to glutmate

Anaesthetic	Measure	Main effect	Interaction	n
Pentobarbitone				
2-5 x 10^{-4} M	Peak	<0.01	NS	6
	Area	<0.05	<0.05	5
10^{-4}	Peak	NS	<0.01	8
	Area	<0.05	<0.05	7
Ketamine				
3.7 x 10^{-4} M	Peak	NS	<0.001	6
3.7 x 10^{-5}	Peak	NS	<0.001	6
Alphaxalone				
1-3 x 10^{-5} M	Peak	NS	<0.01	12
	Area	NS	<0.001	12
Saffan vehicle	Peak	NS	NS	4
	Area	<0.05	NS	4
Metomidate				
1.8 x 10^{-3} M	Peak	NS	<0.001	3
	Area	NS	<0.001	3
All concentrations	Peak	NS	<0.001	9
	Area	NS	<0.001	9

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DISSIMILAR INFLUENCES OF SOME INJECTABLE ANAESTHETICS ON THE RESPONSES OF RETICULO-SPINAL NEURONES TO INHIBITORY TRANSMITTERS IN THE LAMPREY

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1 Intracellular recordings were made from identified bulbar reticulo-spinal neurones in the medulla of lamprey ammocoetes. Responses to iontophoretically applied inhibitory transmitters were measured as changes in membrane potential and input resistance.

2 Dose-dependent alterations in the responses to γ-aminobutyric acid (GABA) and glycine during bath application of injectable anaesthetic drugs were measured; the compounds used were pentobarbitone, ketamine, metomidate and the steroid mixture alphaxalone/alphadolone (Saffan).

3 GABA responses were potentiated by pentobarbitone ($1-3 \times 10^{-4}$ M) and prolonged by ketamine (3.7×10^{-5} M); but depressed by high concentrations (10^{-3} M) of all drugs, as well as by anaesthetic concentrations of alphaxalone ($1-3 \times 10^{-5}$ M).

4 Glycine responses were depressed by alphaxalone ($1-3 \times 10^{-5}$ M) and by supra-anaesthetic concentrations of ketamine (3.7×10^{-4} M) and metomidate (1.8×10^{-3} M). No drug potentiated the glycine responses.

5 In the absence of an effect common to the 4 anaesthetics, it is concluded that neither potentiation nor inhibition of all GABA or glycine responses is an essential feature of anaesthesia. However, effects comparable to those described here may contribute to the overall clinical picture during anaesthesia of higher vertebrates. The findings do not support the notion that all anaesthetic agents act on biological membranes by a single mechanism.

Introduction

Anaesthesia is thought to result from a reversible drug-induced disruption of brain function. Since Sherrington (1906) drew attention to the susceptibility of reflex arcs, attention has focussed on the synapse as the site of action of anaesthetic drugs. It has become apparent that anaesthetics differ in their actions at individual synapses (Larrabee & Posternak, 1952) and that different central nervous pathways have unequal sensitivities (Mark & Steiner, 1958). Indeed some such differential action is required to explain the characteristic clinical states which follow administration of the various drugs (Vickers, Wood-Smith & Stweart, 1978).

In this study we have compared the abilities of four injectable anaesthetic drugs to interfere with the actions of the inhibitory transmitters γ-aminobutyric acid (GABA) and glycine. The drugs were applied to bulbar reticulospinal neurones in the medulla of the lamprey ammocoete, cells readily identifiable and known to possess distinct GABA and glycine receptors (Martin, 1978; Matthews & Wickelgren, 1979). Anatomical and pharmacological analogies have been drawn between these cells and cells in the central nervous systems of higher vertebrates (Shapovalov, 1975; Martin, 1979a).

The anaesthetics, chosen to represent four classes of compound used in clinical anaesthetic practice, were bath-applied to the preparation in concentrations similar to those likely to obtain in the brain during clinical anaesthesia (Richards, 1972; Minchin, 1981). Although anaesthetics could equally interfere with synaptic transmission by a presynaptic action, we have concentrated on postsynaptic mechanisms, applying the putative transmitters directly to the cells and taking steps to preclude transsynaptic effects.

Methods

The preparation

Ammocoetes 8–15 cm long of the species *Lampetra fluviatilis* and *Lampetra planeri* were anaesthetized with neutralized tricaine methane sulphonate (100 μg/l) and then transected caudal to the gills. The rostral portion was divided in the ventral midline and, after tissues covering the ventral notochord had been removed, was pinned out on Sylgard in the experimental chamber, dorsal surface uppermost. A simple

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dissection exposed the bulbar reticulo-spinal cells, clustered on either side of the midline near the centre of the 4th ventricle (Figure 1). The preparation was continuously perfused with a cooled Ringer solution at 6 ml/min, and the recorded temperature in the chamber (capacity, 1 ml) maintained at 8–10°C by the circulation of cooled fluid through an outer jacket.

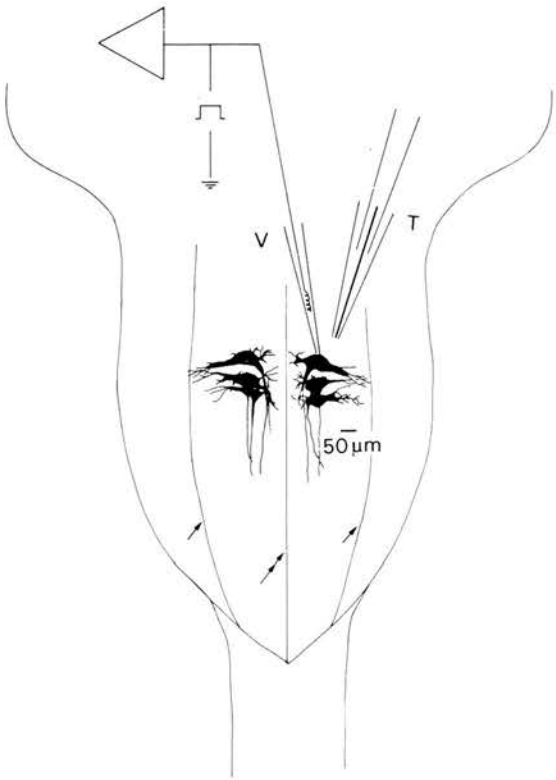

Figure 1 Diagram of the preparation showing the location of micropipettes. Bulbar reticulo-spinal cells lie in the floor of the 4th ventricle between the sulcus limitans of His (arrows) and the median longitudinal sulcus (double arrow) of the ammocoete larva. Positions of recording (V) and iontophoresis (T) electrodes are indicated.

Ringer solutions

The usual composition of the Ringer solution was (mM): NaCl 71, KCl 2.1, $CaCl_2$ 2.6, $MgCl_2$ 15, $NaHCO_3$ 20, NaH_2PO_4 0.18 and glucose 4.0. The pH was adjusted to 7.1–7.2 with HCl and the solution gassed with a mixture of CO_2 5% and O_2 95%. The high concentration of magnesium was used to block the release of endogenous transmitter.

Recording system

Standard intracellular recording techniques were used, with direct visual control of cell penetration. The recording electrode was connected by an Ag/AgCl wire to a high impedance pre-amplifier which incorporated a bridge balance circuit enabling current pulses to be injected for the measurement of input impedance. When filled with 2 M potassium methyl sulphate the glass micropipettes used had d.c. resistances in the range 20–30 MΩ. An Ag/AgCl reference electrode was connected to the bath.

Membrane potentials were displayed on a cathode ray oscilloscope (Tektronix 5113 Dual Beam Storage Oscilloscope) and a permanent record made by a high speed pen recorder (Lectromed MX212). A thermistor probe connected to the pen recorder provided a continuous record of the temperature in the experimental chamber.

Iontophoresis

Three-barrelled assemblies were used for iontophoresis. The micropipettes were back-filled with GABA (0.5 M, pH 2.5), glycine (0.5 M, pH 5.0), or NaCl (0.5 M). GABA and glycine were ejected as cations. NaCl was incorporated as a control in some experiments; in others, application of current pulses of reversed polarity served instead. In all experiments an Ag/AgCl indifferent electrode was placed in the bath for the return of iontophoretic current. Retaining currents were not routinely employed, any leakage of drug was insufficient to cause detectable changes in the membrane potential or resistance of the cells.

The ejection cycle was controlled by a microiontophoresis programmer (Model 160, W-P Instruments Inc.) driven by a Digitimer. The transmitters were applied alternately by currents of the same strength, usually in the range 50 to 90 nA. Pulses lasting between 0.3 and 7.0 s were applied at intervals of 1 or 3 min. The pulse duration was determined for each transmitter individually and then kept constant for the duration of an experiment.

In experiments testing the influence of transmitter concentration on the anaesthetic effect only one transmitter was used. To vary the agonist dose a cycle of iontophoretic pulses of differing duration but the same amplitude was employed. The quantity of drug ejected by an iontophoretic pulse is affected by the immediately preceding pulse, but the use of a fixed pulse sequence ensured that this factor was unchanged during an experiment. Differences between the amounts of the agonist delivered by the test pulses were therefore constant although unknown, so measurements of the responses are comparable but only within an experiment.

The effects of anaesthetics on transmitter responses

Anaesthetics were dissolved in the perfusing Ringer solution and applied to the preparation. No application was continued after 25 min; this limit allowed the responses to recover before cell deterioration supervened. The sequence of iontophoretic pulses continued during the perfusion with anaesthetic. Changes in input resistance associated with the transmitter actions were monitored by the injection of constant hyperpolarizing current pulses (strength 0.5–1.0 nA, frequency 1 Hz) whose duration was long (30 ms) in relation to the time constant of the cell (< 10 ms). Because of the possibility that anaesthetic drugs may interact (Richards & White, 1981), only one was tested on any individual preparation. Most experiments were terminated within 10 h of dissection.

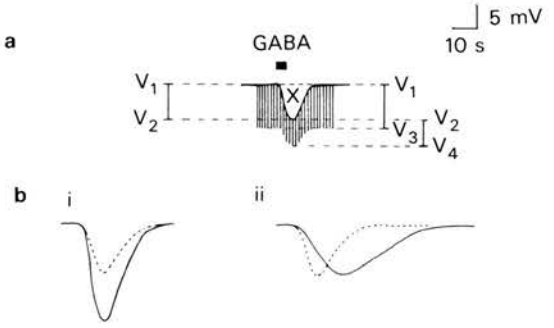

Figure 2 (a) Tracing of a typical response to an iontophoretic pulse of γ-aminobutyric acid (GABA). Vertical deflections (downward) from the resting membrane potential are in response to repeated injections of hyperpolarizing current pulses (1 nA, 30 ms). GABA was applied during the period indicated by the horizontal bar. Features measured: (1) peak change in membrane potential ($V_2 - V_1$), (2) membrane potential change in response to hyperpolarizing current pulses before ($V_3 - V_1$) and during ($V_4 - V_2$) action of GABA. The height of each vertical deflection is directly proportional to the input resistance. Because constant current pulses were used, the fractional change in input resistance at peak GABA effect is

$$\frac{(V_3 - V_1) - (V_4 - V_2)}{(V_3 - V_1)}.$$

(3) area (X) enclosed by the envelope of the membrane potential response. (b) Diagram to illustrate use of measurement of ratio area:peak. Control (- - -) and test (———) responses are superimposed. (i) Simple potentiation of membrane potential response. (ii) Alteration of response profile showing both delayed peak response and slowed recovery. In both (i) and (ii) the area of the response is increased from 9 mV.s (control) to 19 mV.s (test); but whereas in (i) the ratio remains 3.5 s, that in (ii) is increased from 3.5 s (control) to 7.2 s (test).

Drugs

The drugs used were GABA (Sigma), glycine (BDH), tricaine methane sulphonate (MS-222, Sandoz), pentobarbitone (May & Baker), ketamine (Vetalar, Parke-Davis), alphaxalone/alphadolone (Saffan, Glaxovet) and metomidate (Hypnodil, Janssen). The vehicle present in Saffan was kindly donated by Glaxovet. Although the product Saffan was used in the experiments, it will be referred to henceforth as alphaxalone because concentrations are expressed in terms of this constituent.

Analysis of results

The responses to GABA and glycine were essentially similar. Numerical values were obtained for the peak resistance changes as indicated in Figure 2a. A videoplan (Reichert-Jung) was used to measure the time integral of the membrane potential response (area 'X'). Figure 2b shows how the ratio of this area to the peak hyperpolarization distinguishes between an increase in area due solely to an altered peak amplitude and one effected by prolongation of the response. The ratio was calculated in experiments in which pentobarbitone or ketamine were tested, because these drugs altered the GABA response profile.

So that the results of different experiments could be compared, the changes in GABA and glycine responses measured during exposure to anaesthetics were expressed as percentages. Student's *t* test was applied to these standardized data to assess the statistical significance of the anaesthetic effects.

Results

Responses to γ-aminobutyric acid and glycine

Most cells had resting potentials in the range −60 to −65 mV and input resistances > 3 MΩ. The usual response to application of either GABA or glycine was a transient hyperpolarization accompanied by reduction in the input resistance to the cell. A small minority of cells (4 out of 92) showed depolarizing responses to both amino acids. High concentrations of either agonist elicited biphasic hyperpolarizing/depolarizing responses and were therefore avoided.

Effect of anaesthetics on the responses to γ-aminobutyric acid and glycine

Sixty-three ammocoetes were exposed to the anaesthetics: pentobarbitone (19 ammocoetes), ketamine (15), alphaxalone (19), metomidate (10); 10 ammocoetes were tested with the Saffan vehicle alone. No anaesthetic consistently altered the mem-

Table 1 The effects of anaesthetics on the responses to γ-aminobutyric acid (GABA) and glycine

Anaesthetic	*Agonist*					
	GABA			*Glycine*		
	Resistance	*Area*	*Area/Peak*	*Resistance*	*Area*	*Area/Peak*
Pentobarbitone (M)						
10^{-3}	−63 ± 11 (2)	−90 ± 11 (2)		+8 (1)	+3 (1)	
$3–5 \times 10^{-4}$	+13 ± 5 (13)*	+25 ± 10 (11)*	+25 ± 7 (8)**	−15 ± 9 (9)	+4 ± 16 (8)	
10^{-4}	+9 ± 11 (6)	+36 ± 17 (8)	+71 ± 30 (8)*	+27 ± 34 (5)	−4 ± 26 (6)	
Ketamine (M)						
3.7×10^{-3}	−4 ± 15 (2)	+108 ± 49 (2)	+233 ± 0 (2)***	−82 ± 1 (2)**	−86 ± 2.5 (2)*	
3.7×10^{-4}	+16 ± 14 (9)	+124 ± 44 (10)*	+78 ± 17 (10)**	−33 ± 9 (9)**	−23 ± 13 (10)	−8 ± 12 (9)
3.7×10^{-5}	+10 ± 15 (8)	+55 ± 43 (8)	+29 ± 17 (8)	−2 ± 9 (10)	−5 ± 11 (10)	+10 ± 8 (10)
Alphaxalone (M)						
$1–3 \times 10^{-4}$	−58 ± 17 (6)*	−69 ± 30 (4)		−39 ± 9 (4)*	−45 ± 20 (5)	
$1–3 \times 10^{-5}$	−56 ± 10 (8)***	−74 ± 5 (8)***		−36 ± 8 (7)**	−49 ± 11 (7)**	
$1–3 \times 10^{-6}$	−15 ± 4 (3)	−22 ± 11 (2)		−5 ± 5 (6)	+22 ± 18 (3)	
Saffan vehicle	−23 ± 6 (10)**	−21 ± 9 (10)*		−19 ± 13 (6)	0 ± 7 (6)	
Metomidate (M)						
1.8×10^{-3}	−100 ± 0 (3)***	−100 ± 0 (3)***		−73 ± 15 (6)**	−77 ± 17 (6)**	
1.8×10^{-4}	−27 ± 12 (5)	−1 ± 17 (5)		−6 ± 10 (4)	+28 ± 23 (4)	
1.8×10^{-5}	−6 ± 5 (6)	+6 ± 7 (6)		+5 ± 9 (7)	+11 ± 12 (7)	

Saffan vehicle dilution equivalent to 10^{-5}–10^{-4} M alphaxalone.
Tabulated figures are percentage changes in response during application of anaesthetic: mean ± s.e. Numbers of observations in parentheses.
Significant effects indicated (*t* test): *$P<0.05$; **$P<0.01$; *** $P<0.001$.

brane potential and only the highest concentrations of pentobarbitone and metomidate (10^{-3} M) affected the input resistance, causing a small reduction. The alterations in the GABA and glycine responses measured during exposure to the four anaesthetics are displayed in Table 1.

Most experiments were carried out at 8–10°C but the results were qualitatively similar at room temperature and at 2°C (not illustrated).

Pentobarbitone

The effects of pentobarbitone were complex and dose-dependent. Records from typical experiments are displayed in Figure 3. Low concentrations ($1-5 \times 10^{-4}$ M) potentiated the GABA responses in some cells, but only in 8 out of 19 tests did the potentiation exceed 10%. The remaining 11 tests showed little change. Potentiated responses were increased both in amplitude and duration (Table 1). In two preparations the concentration was raised to 10^{-3} M: this caused a reduction in amplitude of the GABA response.

No concentration of pentobarbitone significantly affected the glycine responses (Table 1, and see Figure 3).

Ketamine

Ketamine selectively potentiated the GABA re-

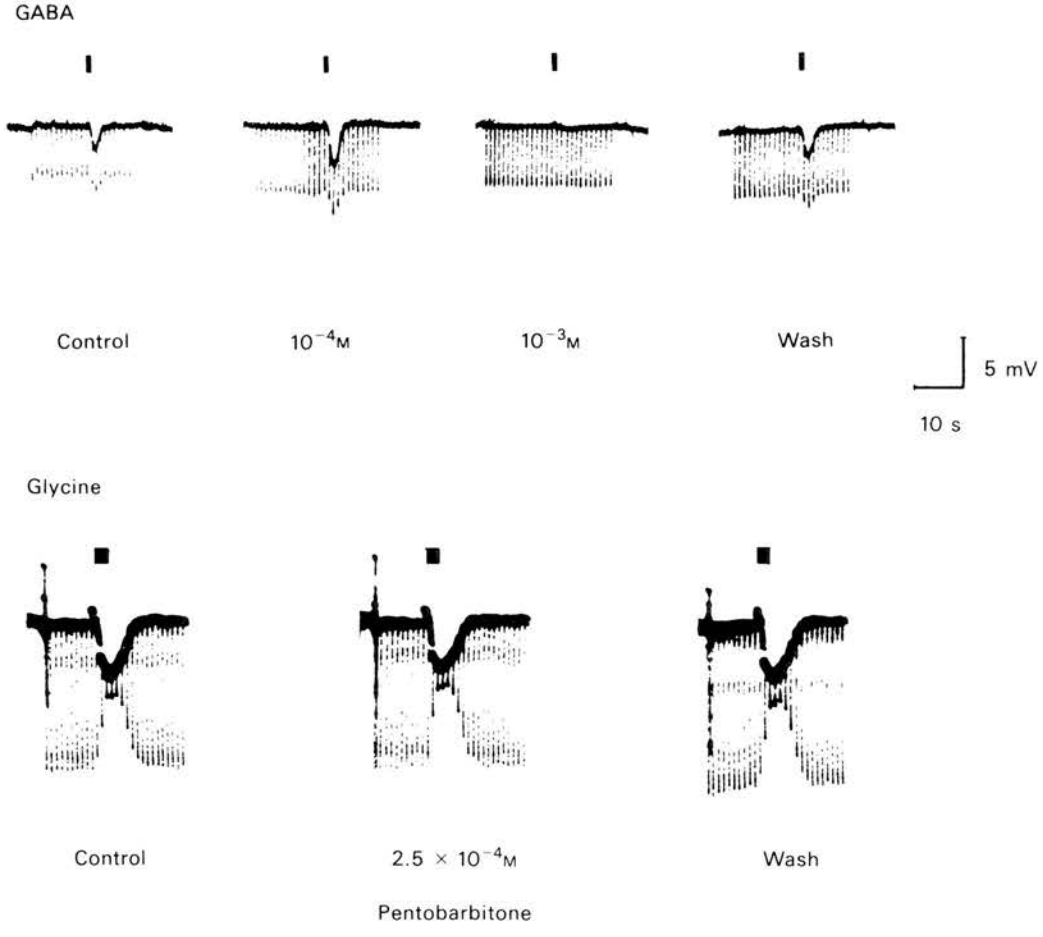

Figure 3 Effects of pentobarbitone on γ aminobutyric acid (GABA) and glycine responses. Records from 2 cells showing: (upper traces), GABA potentiation by 10^{-4} M pentobarbitone and antagonism by 10^{-3} M pentobarbitone (no intervening wash); (lower traces) lack of effect of 2.5×10^{-4} M pentobarbitone on glycine responses. Durations of exposures to pentobarbitone: (upper trace) 13 min (10^{-4} M), 6 min (10^{-3} M); (lower trace) 14 min. Preparations washed for 8 min (upper trace), 14 min (lower trace). Iontophoretic pulses: GABA 0.5 s, 60 nA; glycine 3 s, 200 nA. Hyperpolarizing current pulses: 1 nA (upper traces), 0.7 nA (lower traces).

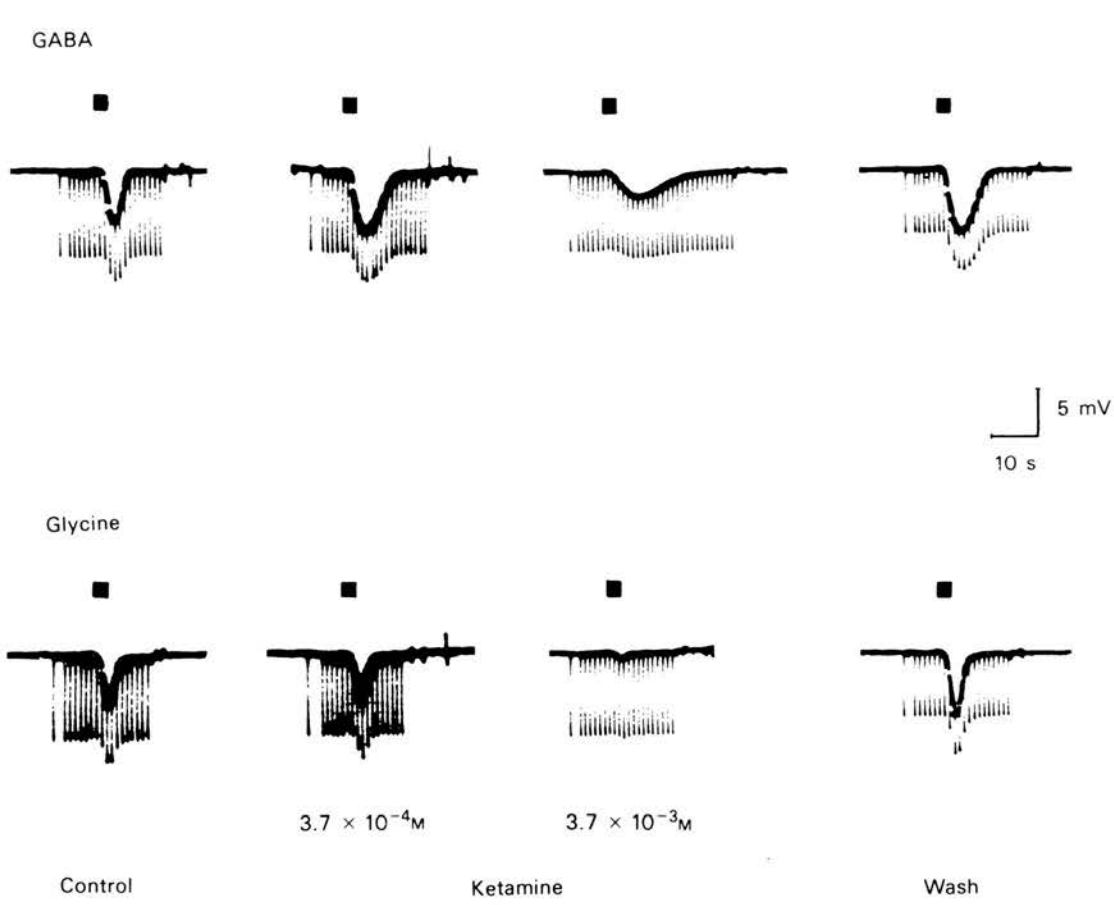

Figure 4 Effects of ketamine on γ aminobutyric acid (GABA) and glycine responses. Records from 1 cell showing GABA potentiation by 3.7×10^{-4} M ketamine and complex effect of 3.7×10^{-3} M (no intervening wash). In this cell glycine responses were unaffected by 3.7×10^{-4} M ketamine but reversibly depressed by 3.7×10^{-3} M. Duration of exposure to ketamine: 8 min (3.7×10^{-4} M), 10 min (3.7×10^{-3} M). Preparation washed for 10 min (upper trace), 11 min (lower trace). Iontophoretic pulses: GABA and glycine 3 s, 60 nA. Hyperpolarizing current pulses: 1 nA.

sponses (Figure 4), an effect consistently detectable at 3.7×10^{-5} M and reaching statistical significance at 3.7×10^{-4} M Ketamine (Table 1). Unlike the potentiation by pentobarbitone, that due to ketamine was almost entirely attributable to prolongation of the response, as is shown by the respective area/peak ratios. Slowing of the onset and offset of the GABA response was most striking at high concentrations of ketamine (3.7×10^{-3} M).

In contrast, Table 1 records a dose-dependent antagonistic action of ketamine on glycine responses. This effect was statistically significant at 3.7×10^{-4} M when the response was measured as the peak change in resistance. Only two cells were tested with ketamine at 3.7×10^{-3} M but in both cases depression of the response was dramatic. No effect of ketamine on the response profile was detected for glycine (e.g. Figure 4).

Alphaxalone

The action of alphaxalone ($> 10^{-5}$ M) was consistently to depress responses to both GABA and glycine (Table 1, and see Figure 5). The commercial vehicle, which contains poly-oxyethylated castor oil, had an antagonistic action in some experiments (7 out of 10 tests using GABA and 3 out of 6 with glycine showed a reduction in response amplitude which exceeded 10%). This latter effect was small, and statistically significant only in the case of resistance measurements on GABA responses.

Metomidate

Metomidate, too, had a dramatic depressant action on both GABA and glycine responses (Figure 6), but only at high concentration (1.8×10^{-3} M). There was

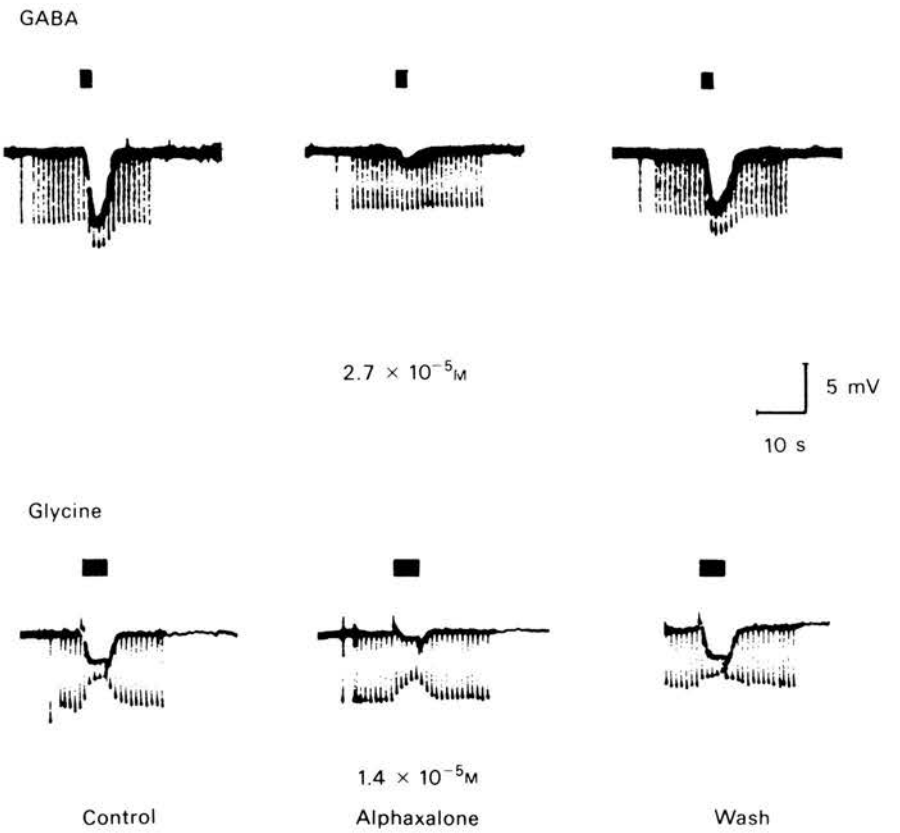

Figure 5 Effect of alphaxalone on γ-aminobutyric acid (GABA) and glycine responses. Records from 2 cells showing reversible depression of GABA (9 min exposure to 2.7×10^{-5} M alphaxalone) and of glycine responses (8 min exposure to 1.4×10^{-5} M). Preparations washed for 22 min (upper trace), 8 min (lower trace). Iontophoretic pulses: GABA 2 s, 30 nA; glycine 5 s, 500 nA. Hyperpolarizing current pulses: 1 nA in upper traces, 0.9 nA in lower traces.

no evidence of potentiation at any concentration (Table 1), nor of alteration in the response profile.

The effect of agonist dose on the modulating influence of anaesthetics

It was recognized that the agonist concentration at the receptors might affect the recorded influence of the anaesthetic drugs. Experiments were therefore performed in which different doses of a single transmitter were applied to a cell.

The same anaesthetic-transmitter combinations as previously described were tested in these experiments, each combination receiving 2–8 tests in between 2 and 4 preparations. Only the potentiation effects were consistently affected by transmitter dose, this being marked particularly with pentobarbitone, which only consistently potentiated GABA responses when short iontophoretic pulses were used. Measurements of peak change in input resistance obtained in one such experiment are shown in Figure 7. In this particular experiment 10^{-4} M pentobarbitone approximately doubled the response to a 0.5 s GABA pulse, whereas the response to a 2 s pulse was increased by only about a quarter. Higher concentrations of pentobarbitone caused a reversible loss of this effect.

Discussion

The lamprey as a model for the study of the effects of anaesthetics

The lamprey brainstem has several features that make it a suitable preparation in which to study the neuropharmacology of anaesthetic drugs. Intracellular recording from identified neurones is practicable and enables changes in input resistance and membrane potential to be measured. This is preferable to

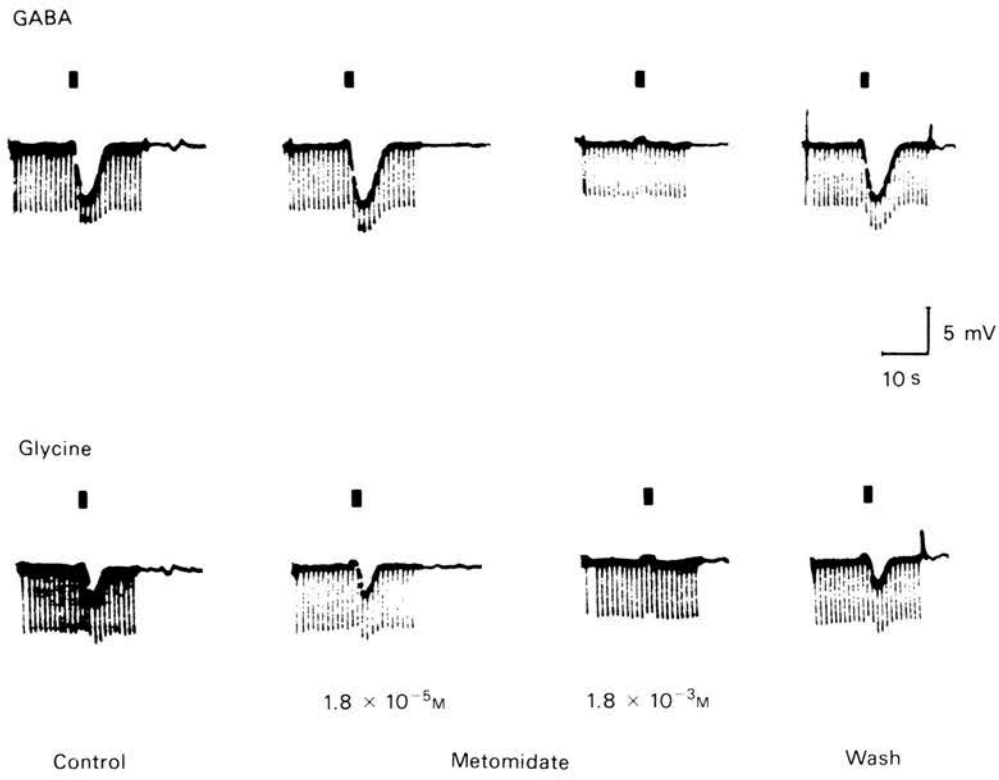

Figure 6 Effect of metomidate on γ-aminobutyric acid (GABA) and glycine responses. Records from 1 cell showing lack of effect of 1.8×10^{-5} M metomidate on GABA and glycine responses. Antagonism by 1.8×10^{-3} M metomidate is total in both cases. Duration of exposures to metomidate (no intervening wash): 10 min (1.8×10^{-5} M), 5 min (1.8×10^{-3} M). Preparation washed for 18 min (upper traces), 17 min (lower trace). Iontophoretic pulses: GABA and glycine 2 s, 300 nA. Hyperpolarizing current pulses: 1 nA.

the use of indirect measures of cell responses, such as changes in spike frequency. The bulbar reticulospinal cells lie close to the surface of the 4th ventricle, covered only by a single layer of ependymal cells; thus the diffusion path for drugs applied in the perfusing Ringer is short (Martin, 1979a, b). In the lamprey brain, blood vessels are confined to the periphery and so neither obscure nor impede access to the cells; visualization is also aided by the absence of myelin.

The neuro-biology of the lamprey has been studied in some detail (for review see Rovainen, 1979). Wickelgren (1977) described diverse sensory inputs to the bulbar neurones and reported that these cells constitute a major motor outflow to the somatic musculature. Any stimulation of the neurones by anaesthetics, effected either directly or via the removal of inhibitory influences, should therefore elicit movements. A similar result is predicted in higher vertebrates, where analogous cells are found (Shapovalov, 1975).

Anaesthetics differ in their postsynaptic effects

We set out to compare the abilities of four anaesthetic drugs to interfere with the responses of reticulospinal neurones to the inhibitory amino acid transmitters GABA and glycine. Many workers have stressed the GABA-potentiating properties of the barbiturates in a range of preparations (e.g. Barker & Ransom, 1978). In our study, as in that of Bowery & Dray (1978), the GABA-potentiating action of pentobarbitone was evident only in a proportion of cells, and showed marked dependence on the agonist dose, being detectable only if low amplitude or short iontophoretic currents were employed.

Ketamine also was found to potentiate GABA responses selectively, but the effect differed from that of pentobarbitone in that it was principally on the time course of the response; both the onset and offset of the GABA response were slowed by ketamine ($> 10^{-4}$ M). Other studies, e.g. Minchin (1981), suggest that this potentiation is not the re-

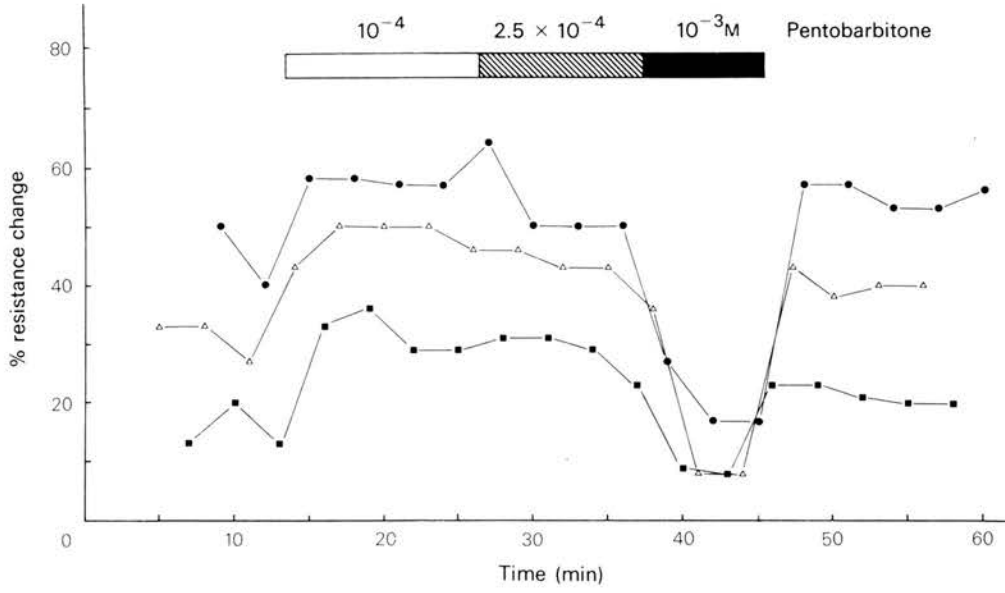

Figure 7 γ-Aminobutyric acid (GABA) induced resistance changes measured in a single cell before, during and after application of increasing concentrations of pentobarbitone. Ordinate scale: GABA-induced resistance change (%); abscissa scale: time after pulse sequence started (min). GABA pulses (amplitude 60 nA) applied for 0.5 s (■), 1.0 s (△) and 2.0 s (●) in repeated sequence. Pentobarbitone 10^{-4} M approximately doubled the response to a 0.5 s GABA pulse whereas the response to a 2.0 s pulse increased by only about a quarter. Higher concentrations of pentobarbitone reversibly eliminated the potentiation.

sult of altered GABA uptake mechanisms. We are unaware of any other studies using ketamine that are directly comparable with ours.

In contrast, neither alphaxalone nor metomidate caused significant potentiation of GABA responses at any concentration tested; instead, both drugs produced parallel depressions of the responses to GABA and glycine. There are no previous reports of the effects of either anaesthetic on responses to the inhibitory transmitters; but etomidate, an imidazole derivative closely related to metomidate, has been studied: it is reported to be similar in action to pentobarbitone, selectively potentiating GABA responses at low doses (10^{-5} M) but having a depressant effect at high doses (10^{-3} M). As with the barbiturates, the potentiating action has been reported to show stereo-specificity (Hill & Taberner, 1975; Huang & Barker, 1980). We have not tested the optical isomers of metomidate separately, but those of etomidate have been shown to differ in their pharmacological actions (Hill & Taberner, 1975).

Although published information about the actions of alphaxalone/alphadolone on inhibitory mechanisms is lacking, Pennefather & Quastel (1980) described alterations in endplate currents recorded at the neuromuscular junction; and Smaje (1976) and Richards & Smaje (1976) reported depressed responses to acetylcholine and glutamate in the olfactory cortex.

Relevance to clinical anaesthesia

Although the reticulo-spinal cells are not concerned with consciousness, the effects we have described are likely to operate during anaesthesia and may be related to side effects produced by the drugs. Our results are hard to reconcile with a unitary biophysical hypothesis of anaesthetic action. Such a hypothesis predicts similarity of action of different anaesthetic compounds (Metcalfe, Hoult & Colley, 1974), however exerted, and this we have not observed. The concentrations at which the anaesthetics have been tested in this study include those likely to obtain in the brain during clinical anaesthesia (Table 3), and were themselves capable of inducing anaesthesia in lamprey ammocoetes (Table 2, and see appendix).

Our finding that neither GABA nor glycine responses were potentiated by alphaxalone and metomidate lends no support to the contention that inhibitory mechanisms are enhanced during anaesthesia. However, that idea derives largely from studies in which barbiturates or volatile anaesthetic agents have been used (e.g. Eccles, Schmidt & Willis,

Table 2 The effects of injectable anaesthetic drugs when bath-applied to intact lampreys

Drug	*Concentration* (M)	*Stage of anaesthesia reached after various time intervals*			
		2 min	5 min	10 min	30 min
Pentobarbitone	10^{-3}	—	—	I	I
Ketamine	3.7×10^{-5}	—	I	II–1	II–1
	3.7×10^{-4}	I	II–1	II–2	II–2
	3.7×10^{-3}	II–1	II–2	II–2	III/IV
Alphaxalone	2.7×10^{-6}	—	—	I	II–2
	2.7×10^{-5}	II–1	II–2	II–2	
	2.7×10^{-4}	II–2	III	III/IV	
Metomidate	1.8×10^{-5}	—	—	I–2	I–2
	1.8×10^{-4}	—	II–1	II–2	II–2
	1.8×10^{-3}	II–2	II–2	II–2	

Drugs dissolved in river water (unbuffered); times measured from moment of transfer of ammocoetes to anaesthetic solution. Classification of anaesthesia after McFarland (1959), tabulated stages are the averages for the group (3–8 ammocoetes).

1963), and those results are not contradicted by our experiments. There is no evidence that GABA and glycine receptors on the reticulo-spinal cells of the lamprey differ pharmacologically from those in higher vertebrates (Martin, 1979a; Nistri & Constanti, 1979).

Only with alphaxalone was depression of GABA and glycine responses seen at concentrations likely to be encountered during clinical anaesthesia in fish (Table 3) and higher vertebrates. Should a comparable depression of inhibitory mechanisms underlie the excitatory phenomena commonly observed during anaesthesia with alphaxalone/alphadolone in mammals, a logical preventive treatment would be premedication with a GABA-potentiating agent. It is interesting that there are clinical reports of a reduced incidence of tremors and purposeless movements when diazepam is used as a pre-medicant before anaesthesia with etomidate (Holdcroft, Morgan, Whitwam & Lumley, 1976) or alphaxalone/alphadolone (Vickers *et al.* 1978). The benzodiazepines as a group are known to potentiate the actions of GABA (Curtis, Lodge, Johnston & Brand, 1976).

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Appendix

Abilities of the drugs to anaesthetize lampreys (Table 2)

Experiments were carried out to ensure that the drugs used in the iontophoresis experiments were capable of inducing anaesthesia in intact lampreys. These experiments give an indication of the concentrations required but do not provide an accurate estimate of the concentration any anaesthetic reached in the brain. The behaviours of groups of 3 ammocoetes immersed in anaesthetic solutions were compared with that of an untreated group. Anaesthesia was assessed according to the criteria of McFarland

Table 3 Estimated brain concentrations of anaesthetics during clinical use in fish

Anaesthetic	*Dose*	*Brain Conc.* (M) (estimate)	*Effect on fish*	*Ref.*
Pentobarbitone	30 mg/kg i.p.	1.1×10^{-4}	Sedation	1
	48 mg/kg i.p.	1.8×10^{-4}	Apnoea	1
Alphaxalone	18 mg/kg i.p.	8.1×10^{-5}	Surgical anaesthesia	1
Ketamine	130 mg/kg i.m.	9.5×10^{-5}*	Surgical anaesthesia	1
Metomidate	7.5 mg/l (bath)	2.8×10^{-5}*	Surgical anaesthesia	2

*Upper limit. Ref. 1 = Oswald (1978), 2 = Stuart (1981).
Assumptions(Minchin, 1981): rapidly acting compounds (alphaxalone, metomidate) distributed in E.C.F. (−30% body weight); slowly acting compounds (pentobarbitone, ketamine) distributed in total body water (−66% body weight); protein binding 10% for alphaxalone and pentobarbitone.

(1959). The anaesthetics were dissolved in river water to the same concentrations as were employed in the iontophoresis experiments and each drug was tested on a minimum of 6 ammocoetes. Most of the experiments were conducted at room temperature (water temperature 15–17°C); but alphaxalone was also tested at 4°C, with similar results. It is recognized that both the rate of induction and the depth of anaesthesia in fishes are affected by temperature, the induction time commonly being shorter at higher temperature (discussed by McFarland, 1959).

Ketamine, alphaxalone and metomidate, when used at concentrations that affected the transmitter responses, induced similar behavioural states that were comparable to those McFarland (1959) reported in fish. A transient period of intense hyperactivity immediately after the introduction of high concentrations of metomidate or ketamine was attributed to the acid reaction of these solutions. A similar reaction of fish to the anaesthetic tricaine methane sulphonate has been described (e.g. Ohr, 1976). No such hyperactivity was noted following the introduction of pentobarbitone or alphaxalone.

The anaesthetic effect of alphaxalone ($> 10^{-5}$ M) stood out because of the rapidity of its onset coupled with the slow recovery; after the ammocoetes were removed from 2.7×10^{-4} M alphaxalone to fresh water it was an hour before there was slight movement in response to tail squeezing. During recovery there was a period when non-propulsive and rolling movements were observed, but frank convulsions were not elicited. In contrast, the lampreys were swimming within 30 min of their removal from 10^{-3} M metomidate. Recovery from high concentrations of ketamine was also slow, but was quiet in comparison to that of ammocoetes exposed to 3.7×10^{-5} M ketamine which was characterized by a period of apparent excitement.

The finding that bath application of even a high concentration of pentobarbitone induced only mild sedation, is consistent with reports that bath application of the barbiturates is an unsatisfactory method of anaesthetizing fis(see McFarland, 1959).

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